STUDIES ON THE FUNCTION OF THE HUMAN ADRENAL CORTEX IN OBESITY AND CUSHING'S SYNDROME

by

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Part I

Introduction

General Introduction

It is now well established that either primary or secondary effects of the adrenocortical hormones affect the kidney function, sweat and saliva, cardiovascular system, nervous system, muscle function, carbohydrate metabolism, protein metabolism, fat metabolism, vitamin metabolism, electrolyte and water metabolism, lymphoid tissue, reticuloendothelial and mast cells, blood and bone marrow, malignancy, bone, skin and hair, appetite, resistance to damage, cellular reaction to inflammation and injury, sensitivity and antigen-antibody reactions, susceptibility to infection, thyroid gland, gonads and lactation (Pincus, 1962). Thus, it seems that there are few, if any, tissues or bodily processes which are not influenced by adrenocortical hormones.

However, we owe our present-day concept of the importance of the adrenal cortex to the early observation of Addison (1855) who described the clinical features of the disease bearing his name, and who suggested that these glands are essential to health and that death occurred in their absence.

Soon after, Brown-Séquard (1856) proved the vital importance of the adrenal glands by his studies of the effects of bilateral adrenalectomy in experimental animals. But it was not until the beginning of this century that serious attempts were made to prolong the life span of adrenalectomised animals by injection of the adrenocortical extract.

The success of these experiments started an avalanche of investigations aiming at isolation and chemical identification of the active principle. Thus in 1930 an extract of the adrenal cortex was prepared simultaneously by two groups of workers; Hartman and Brownell, and Swingle and Pfiffner, and soon after, isolation of the individual active hormones began . It was the work of

Wintersteiner,/

Wintersteiner, Kendall, Reichstein and their associates (Wintersteiner and Pfiffner, 1936; Mason et al, 1937; Mason et al, 1938; Reichstein and Schoppee, 1943) that made possible the chemical identification of a large group of the adrenocortical hormones and shed more light on their physiological significance. Since then, more than sixty adrenocortical hormones have been isolated and identified.

Semiquantitative tests for the assessment of adrenocortical function, by making use of the laboratory animals, started in 1938 when Anderson and Haymaker prolonged the life of adrenalectomised dogs by injecting them with sera from patients suffering from Cushing's syndrome; adrenalectomised dogs injected with sera from normal subjects acted as controls. Other biological tests were also used in these early days, such as assessment of the degree of protection from exposure to cold after injection of serum, the use of urinary extracts to measure the survival time of adrenalectomised rats on exposure to cold, the degree of deposition of liver glycogen in fasting adrenalectomised rats, the extent of fall of circulating eosinophils in the adrenalectomised mice or the degree of sodium retention and potassium excretion in the adrenalectomised rats.

Chemical tests for the quantitative estimation of adrenocortical function started in 1945 when Talbot and co-workers estimated the urinary corticosteroids by a colorimetric reaction and Hemphill and Reiss (1947) using the same colorimetric reaction to estimate corticosteroids in blood. These methods lacked specificity but, since then, marked advances in the field of chemical analysis and estimation of adrenocortical hormones have occurred with the result that a great number of simple and reliable tests of adrenocortical function have been introduced. These tests are nowadays of an immense help to both the clinician, helping him to combat the various adrenocortical dysfunctions, and the biochemist, helping him to clarify further the role of the adrenal cortex in human life.

Chapter I: The Human Adrenal Cortex and its Hormones

Development of the adrenal cortex

The cells of the adrenal cortex arise from the celomic mesoderm of the posterior abdominal wall and condense into a small cluster of acidophilic cells between the root of the mesentry and the genital ridge. Cells of the genital ridge may, therefore, be included in the cortex, and this explains the occurrence of androgenic and estrogenic activity in the adrenal cortex later in life, as well as the frequent occurrence of adrenal rests in the testes, ovaries, spleen and in other tissues (Graham, 1953). It is estimated that as many as twenty percent of normal subjects have accessory adrenocortical tissue which is potentially capable of secreting steroid hormones (Forsham, 1962). Later, basophilic cells gradually surround and permanently replace the acidophilic ones. Although the adrenal cortex and medulla form one anatomical structure in the adult human subjects, yet the medulla has a completely different origin, namely from ectodermal neurogenic cells migrating down from the neural crest. Secretions and physiological actions of the adrenal cortex and medulla are thus completely different; the cortex secretes steroids and the medulla secretes catecholamines, and no direct functional relationship between them has been proven in man.

Morphology, histochemistry and functional zonation of the adrenal cortex

The normal human adrenal glands weigh between 5.0 and 7.0 grams each (Forsham, 1962). However, Studzinski and co-workers (1963) noted a difference between glands removed at autopsy, the mean weight of each of which is 6.0 grams, and those removed at operation, the mean weight of each of which is significantly less, namely 4.0 grams.

The human adrenal cortex is composed of three zones surrounded by a capsule. The first zone lining the capsule is the zona glomerulosa, which varies in thickness in normal glands from a prominent layer to almost nothing (Symington, 1961) but which usually constitutes a tiny fraction of the cortex. The cells are arranged in cords which form a basket-work parallel to the surface of the gland and there is no sharp division between this zone and the underlying zona fasciculata. The cells of the zona glomerulosa contain some lipid and are rich in ribonucleic acid, mitochondria and dehydrogenase enzymes (Symington, 1962). It is well established now that the zona glomerulosa is concerned mostly with aldosterone secretion.

The zona fasciculata, the widest zone, is composed of radially oriented strands of cells separated by capillaries. Under basal conditions, these cells are filled with large lipid globules which are removed during preparation of paraffin sections leaving clear spaces, and thus they were called vacuolated or "clear cells". Since these lipid droplets possess optical and staining properties characteristic of cholesterol esters and cholesterol, it has been concluded that such substances are present in abundance as components of the lipid droplets (Symington, 1962). The cells of this layer are poor in ribonucleic acid, alkaline and acid phosphatase, dehydrogenase enzymes of the citric acid cycle and contain only a few mitochondria scattered in their cytoplasm (Symington, 1961, 1962).

The

The clear cells of the zona fasciculata change abruptly into the "compact cells" of the zona reticularis which are arranged in alveoli separated by sinusoids. These cells are small and have scanty lipid droplets and their cytoplasm is eosinophilic. They are rich in ribonucleic acid, alkaline and acid phosphatases, dehydrogenases of the citric acid cycle and packed with mitochondria. Mitotic division has been demonstrated only in the compact cells of the cortex.

On section, the outer zone of the cortex is yellow in colour and the inner zone corresponding to the reticularis is brown.

The extensive study of Symington, Grant and their associates of the histochemistry of the adrenal cortex has contributed a great deal to the present-day concept of the relationship between the histochemistry and the functional zonation of the adrenal cortex. Thus, in 1962, Symington reviewed previous evidence and concluded that stress caused by conditions such as severe burning, coronary thrombosis or severe infection, leads, within twelve to twenty-four hours, to 'focal lipid depletion' of the zona fasciculata. During this period, the gland elaborates and secretes large amounts of steroid hormones. If stress continues, complete lipid depletion occurs and the zona fasciculata and the zona reticularis become a uniform zone of enzyme-rich but lipid-depleted compact cells. These changes may be compared to those produced by administration of ACTH (Symington, 1961) under the effect of which clear cells change to compact cells. Lipid depletion after ACTH occurs in a regular manner and involves the inner aspect of the whole of the zona fasciculata. With large doses of ACTH, depletion extends outwards to affect a complete change in the whole cortex and with smaller doses, cellular transformation from clear to compact/

compact cells involves only half of the fasciculata, but it does so in a diffuse and not a focal manner. Under the effect of ACTH also, it was noticed that little or no change in the histochemistry of the zona reticularis occurs while the fasciculata content of alkaline and acid phosphatases, dehydrogenases and ribonucleic acid become abundant, mitochondria increase, the ratio of cortisol to corticosterone in the adrenal vein effluent increases and the 11β-hydroxylase of the whole gland increased markedly.

From these and previous observations, it was concluded that, firstly, the zona reticularis in man is the site of production of the C-21 steroids (with the exception of aldosterone, which is believed to be secreted by the glomerulosa zone) and probably also the C-19 androgens and the C-18 estrogens; secondly, the clear cells of the zona fasciculata are a storage zone for steroid precursors (cholesterol and cholesterol esters); and thirdly, in conditions of stress and after ACTH stimulation the steroid precursors in the cells of the of the zona fasciculata, nearest to the zona reticularis, are used for steroid biosynthesis in the cells of the zona fasciculata; and lastly, this results in time in the morphological, histochemical and enzymic changes previously described. However, these workers (Griffiths et al, 1963) were able to prove very recently that 11β-hydroxylase activity and free cholesterol content were equal in both clear and compact cells and thus the increased 11β-hydroxylase activity previously observed in glands of ACTH-treated patients could not be explained on the basis of increased number of compact cells, but could be attributed to the compact cells newly formed, under the influence of ACTH, from clear cells at the fascicular-reticular border. Also, 11β-hydroxylase activity could not obviously be related to the number of mitochondria. These/

These workers also isolated 11β-hydroxy-androst-4-ene-3,17-dione from both the reticularis and fasciculata zones, a finding which was not in keeping with these zonation theories which attributed adrenal androgen production to the reticularis zone alone. Accordingly, these workers modified their earlier hypothesis to include the view that clear as well as compact cell zones contribute to a daily basal secretion of cortisol and adrenal androgens with principal effect of ACTH on clear cells, and that as far as steroid biosynthesis is concerned, it is possible that the functioning of the clear cells of the zona fasciculata in man is a safety mechanism designed to provide additional cortical hormone in emergency; if the emergency is prolonged, the functioning of the clear cells may give time for the cortex to increase enzymes and structures required to meet the continued demand for cortical hormones. Thus the previously observed changes in ribonucleic acid and phospholipid, and the well established increased uptake of amino acids by the adrenal cortex in response to ACTH would be in keeping with this theory.

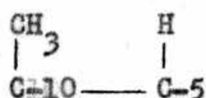
Rich arterial blood supply reaches the suprarenal cortex from the aorta, phrenic artery, renal artery and sometimes also from the ovarian or internal spermatic artery. Some sixty arterioles may be found, and these break up into sinusoids which traverse the substance of the adrenal and end in large venous lacunae in the medulla from which blood is collected in a single large vein, the adrenal vein, and in a number of smaller ones. Thick longitudinal muscle bundles are present in the wall of the central adrenal vein, these are evenly concentric in the centre of the gland, but on either side they become typically eccentric. Capillaries or venules coming from the cortex enter the lumen of the central vein only between the longitudinal muscle bundles and never through the thin/

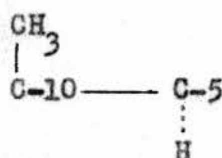
thin side of the vein wall (Symington, 1962). The importance of the hemodynamics of this portal circulation lies in the fact that blood-borne diseases, like tuberculosis, tend to destroy the adrenal gland more extensively, while spontaneous atrophy affects mostly its inner layers, which are poorly oxygenated. Moreover, no secretory nerves have been demonstrated in the adrenal cortex, but only autonomic ones which either directly pass on to the wall of the central vein or pass without branching into the medulla. The importance of the musculature and innervation of the central vein will be discussed later in connection with regulation of secretion of hormones by the adrenal cortex.

Biochemistry of the adrenocortical hormones

All steroid hormones are ultimately derivatives of the cyclopentanoperhydrophenanthrene ring or its methylated derivative sterane (see figure 1). The hormones of the adrenal cortex contain either 21, 19 or 18 carbon atoms. C-21 hormones comprise the corticosteroids and precursor hormones, often referred to as 'progesteroids', C-19 derivatives comprise the androgens and C-18 derivatives, after aromatisation of the A ring, yield estrogens. Corticosteroids are often subdivided into two sub-groups; 'glucocorticoids' which are hormones particularly active in carbohydrate metabolism and relatively less active in electrolyte metabolism, the most important of which by far in man is cortisol (Mason and Sprague, 1948; Bush and Sandberg, 1953; Romanoff et al, 1953; Hudson and Lumbardo, 1955), and 'mineralocorticoids' which are hormones having weak action on carbohydrate metabolism but exert a pronounced effect on electrolyte and water metabolism, the most potent of which is aldosterone.

Sterioisomerism occurs among the hormones of the adrenal cortex. This is designated as 'cis' and 'trans' when relating ring A and B; C and D are always in the 'trans' position in biological compounds. Substituents in ring A and B are named with reference to the methyl group at C-10 which is assumed to be above the plane of the ring. Beta groups lie above and alpha groups below the plane; the former link is represented by a solid line and the latter by a broken line





"beta" "alpha"

In the adrenal steroids, all active compounds carry a 21"β"-alpha ketol group and a 17 alpha-hydroxy group, whereas the 11-hydroxyl, a hallmark of adrenal origin,/

origin, is in the beta position. However, a hydroxyl group, and not a ketone group, at C-11 is necessary for activity of the hormone (Bush, 1956; Bush and Mahesh, 1958) all active corticosteroids possess a double bond between C-4 and C-5 and an oxygen atom at C-3, an arrangement commonly referred to as Δ^4-3α-ketone group.

'Trivial names' are frequently used instead of the 'chemical names' of these hormones (see appendix II).

Biosynthesis of the adrenocortical hormones

This was studied in the past by either perfusion of the isolated suprarenal gland and isolating the hormones emerging from the main vein (Hechter et al, 1950), by cannulation of the suprarenal central vein during operations or by isolation and identification of steroid hormones from adrenal slices or homogenates (Plager and Samuels, 1952; Wettstein, 1954). Since the introduction of isotopes, however, more accurate observations can be made either *in vitro*, by incubation of adrenocortical slices with labelled steroid substrates and isolation of the labelled transformation products, or *in vivo* by injection of the labelled precursor and isolation from the blood of its transformation products.

In the cortex, cholesterol is formed from acetate (acetate → aceto-acetate → Mevalonate → squalene → lanosterol → zymosterol → desmosterol → cholesterol).

Cholesterol is believed to be the precursor of all steroid hormones, and the administration of an inhibitor of cholesterol biosynthesis, triparanol (MER - 29), which blocks cholesterol formation by inhibiting reduction of the 24,25 double bond of desmosterol, results in a significant diminution of cortisol and aldosterone secretion (Melby et al, 1961). Nevertheless, *in vitro* studies showed that adrenocortical steroids could be produced directly from acetate without the intermediate formation of cholesterol (Melby et al, 1961; Forsham, 1962) and accordingly, although corticosteroid synthesis from cholesterol could be sometimes blocked, secretion rates and plasma levels of corticosteroids will not be affected, probably by diversion of corticosteroid synthesis to a non-cholesterol pathway (Melby et al, 1961; Ney et al, 1962).

A/

A number of enzymes affect the biosynthetic transformations to the various corticosteroids. These include specific hydroxylases, which introduce hydroxyl groups, dehydrogenases, which affect removal of hydrogen atoms, reductases, which change ketone groups into hydroxyl groups, and isomerases. These specific enzyme systems include co-factors such as DPN or TPN, located in the microsomes, mitochondria or cytoplasm.

The first step in the biosynthesis of corticosteroids is the formation of (Δ^5-pregnenolone) from cholesterol by side-chain scission, possibly through the intermediate formation of 20-hydroxy-cholesterol followed by cleavage of the side chain. This step is believed to be enhanced by corticotrophin. Progesterone is then formed from Δ^5-pregnenolone. This step is catalyzed by two enzymes; 3β-hydroxysteroid dehydrogenase found in the microsome and DPN or TPN dependent, and Δ-^{5}, Δ^4 isomerase in the supernatant. The different biosynthetic pathways of corticosteroids are shown in figure 1. It is desirable, however, to sum up the present-day concept of cortisol biosynthesis. The main pathway from progesterone involves 17-hydroxylation to 17α-hydroxy-progesterone followed by 21-hydroxylation to 11-deoxycortisol and finally 11-hydroxylation to cortisol. A second possible pathway is through 11-hydroxylation of progesterone to 11β-hydroxyprogesterone followed by 17-hydroxylation to 21-deoxycortisol and finally 21-hydroxylation of the latter compound to cortisol. A third hypothetical pathway would be through 21-hydroxylation of progesterone to 11-deoxycorticosterone followed by 11-hydroxylation to corticosterone and finally 17-hydroxylation to cortisol. In these major and hypothetic pathways of cortisol biosynthesis the enzyme 3β-hydroxysteroid dehydrogenase acts prior to 11β-, 17α- and 21-hydroxylations. But the recent discovery of 21-hydroxypregnenolone in human urine (Pasqualini and/

and Jayle, 1962) suggested the existence of an alternate pathway for cortisol biosynthesis, in which the enzyme 3β-hydroxysteroid dehydrogenase either acts after 21-hydroxylation of pregnenolone to give deoxycorticosterone or acts after both 21- and 11β-hydroxylation to give corticosterone.

Nearly fifty steroids have been isolated so far from the adrenal cortex, but only nine have been shown to be secreted into the blood, the remainder being intracellular intermediates. The nine hormones secreted into the blood include the progesteroid, progesterone; the estrogen, estradiol; the androgens, DHA, Δ^4-androstenedione and 11β-hydroxyandrostenedione and the corticosteroids, cortisol, corticosterone, aldosterone and 11-deoxycorticosterone.

The characteristic features essential for corticosteroid hormonal activity are firstly the steroid nucleus, secondly the oxygen attached to C-3, thirdly the double bond between C-4 and C-5, and fourthly the side-chain on C-17 (Cope, 1961). In some hormones other additional features are important, e.g. a hydroxyl group at C-17 in glucocorticoids, a hydroxyl group at C-11 in cortisol, an aldehyde group at C-18 in aldosterone, etc.

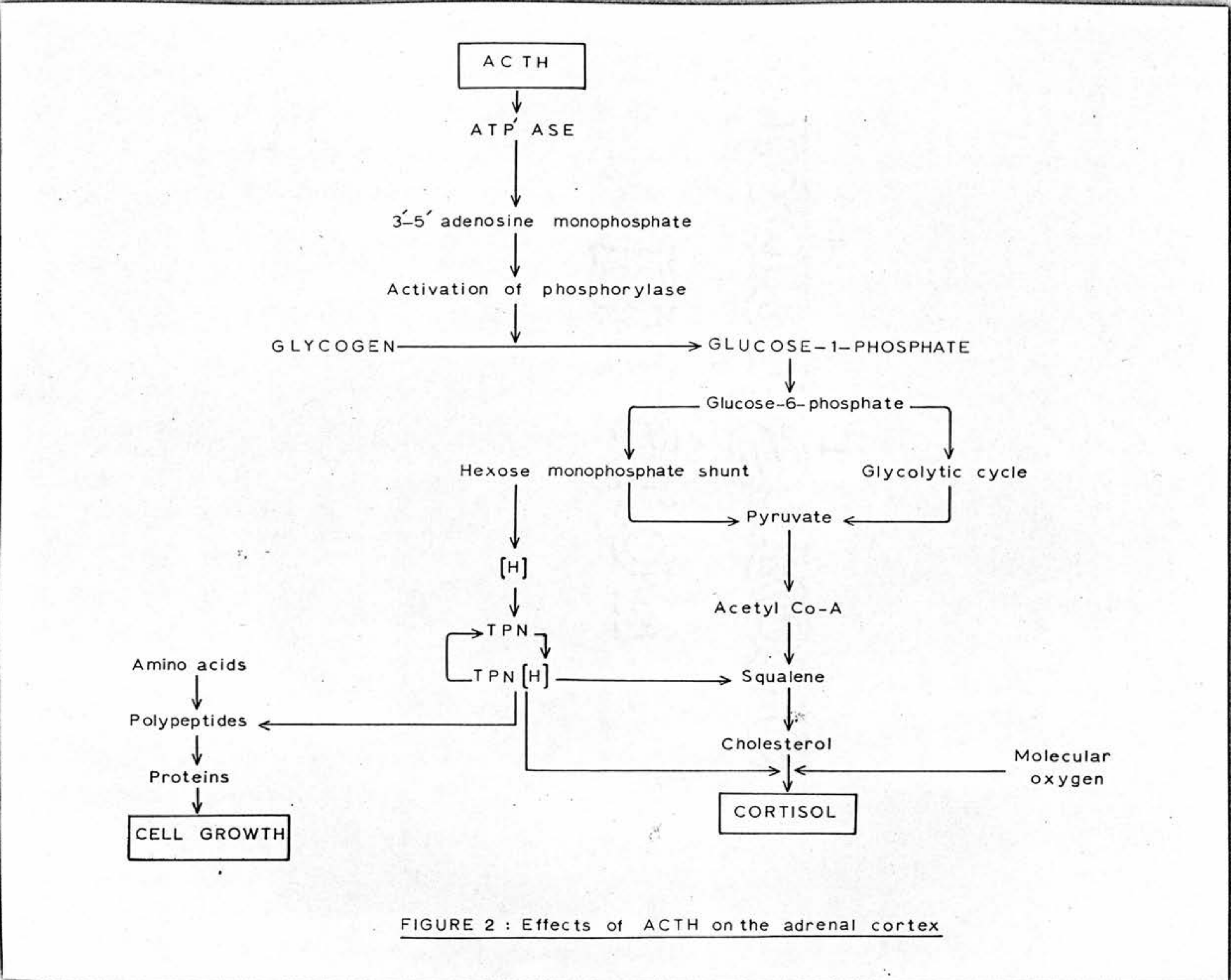


FIGURE 2 : Effects of ACTH on the adrenal cortex

Regulation of cortisol secretion

~~Present~~ Recent investigations have helped clarify the mechanisms involved in the synthesis and release of corticosteroids as well as maintenance of structure and growth of the adrenal cortex cells. The concentration of total cholesterol is higher in the adrenals than in any other tissue but the nervous system; 80-90% of this cholesterol is esterified. The adrenals synthesise cholesterol and its esters freely and it also receives a good deal from the plasma. Also, the adrenals have relatively high concentration of glycogen in the zona fasciculata and zona reticularis but very little in the zona glomerulosa. Both severe stress and injection of ACTH cause pronounced decrease in the quantity of cholesterol and rapid depletion of glycogen from the adrenal cortex. The adrenal lipids are not decreased significantly until the glycogen is almost gone. These changes are associated with a maximal release of adrenocortical hormones particularly cortisol and corticosterone. Bearing in mind that glucose-6-phosphate dehydrogenase is also plentiful in the zona fasciculata and zona reticularis but low in the zona glomerulosa, the following sequence of events seems significant in synthesis and secretion of cortisol from the adrenal cortex. ACTH stimulates formation of 3'-5'-cyclic adenosine monophosphate, which, with various co-factors, leads to the activation of phosphorylase. The latter accelerates glycogenolysis, thus increasing glucose-6-phosphate and consequently acetate and cholesterol synthesis. Since a very active hexosemonophosphate shunt is present in adrenal tissue, the amount of glucose-6-phosphate metabolised via this pathway is increased, with the result that more TPNH is produced which accelerates splitting of the cholesterol side chain and reduction of certain steroids leading to increased synthesis of corticosteroids.

ACTH/

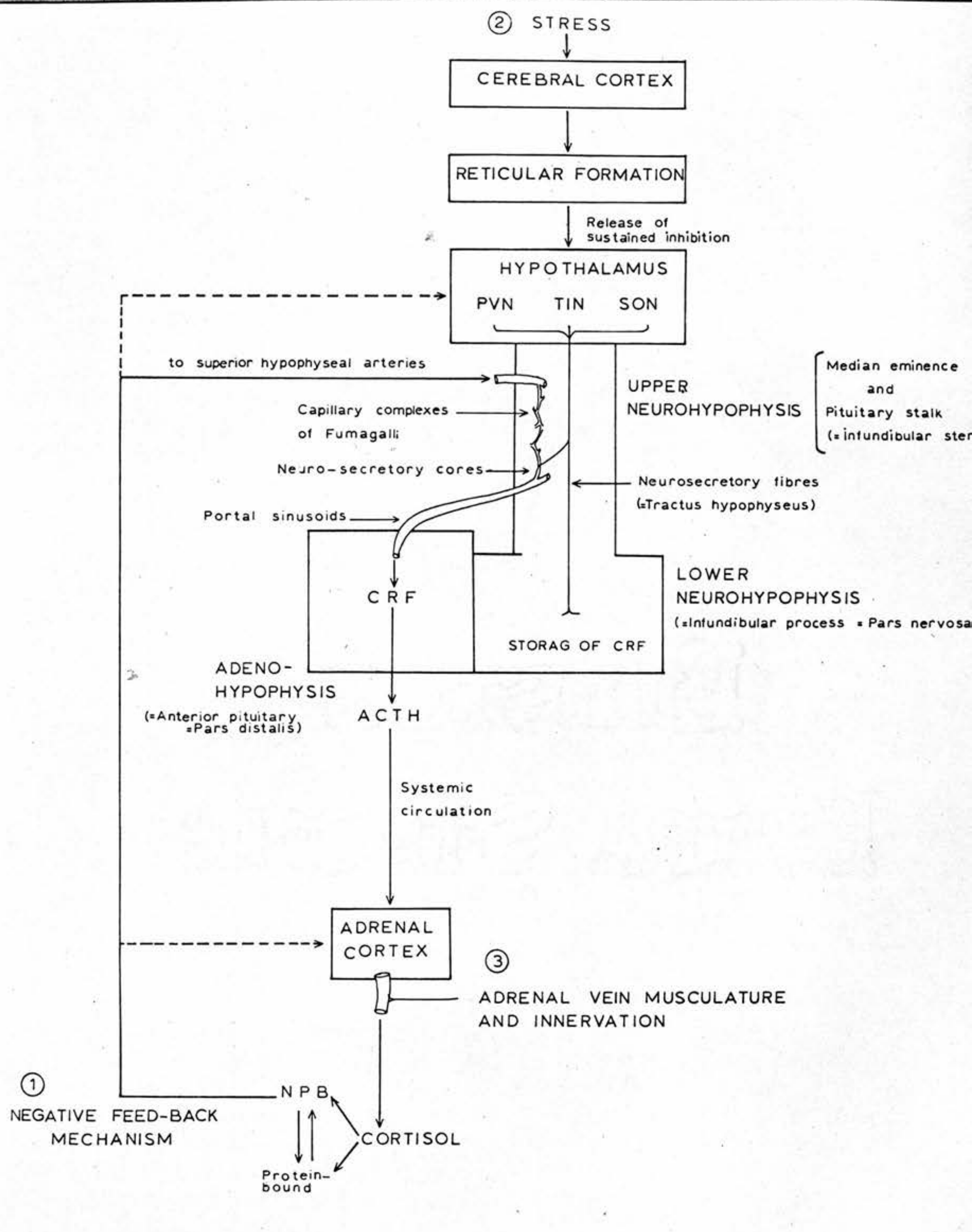


FIGURE 3 : Regulation of cortisol secretion

ACTH also enhances adrenocortical cell growth. These postulated actions of ACTH are illustrated in figure 2. In the human being ACTH, secreted by the basophil "beta prime" cells of the anterior pituitary, is responsible for these changes. It has long been established that the adrenal cortex is under the control of the pituitary gland and that the secretion of ACTH by the pituitary gland is inversely related to the level of circulating glucocorticoids (Sayers and Sayers, 1948; Sayers, 1950). No secretory nerves have been demonstrated in the adrenal cortex. It was also suggested a long time ago that the anterior pituitary is at least partially under the control of the hypothalamus (de Groot and Harris, 1950; Hume and Wittenstein, 1950).

The principal mechanism responsible for and regulating the secretion of cortisol from the adrenal cortex is called the "servo mechanism" or the "negative feed-back mechanism" (figure 3) whereby cortisol is secreted from the adrenal cortex into the circulation, and its level in the blood which reaches the anterior pituitary specifically controls the synthesis and release of ACTH. But this blood, derived from the superior hypophyseal artery, reaches the capillary complexes of Fumagalli in the upper neurohypophysis and passes to the portal sinusoids which connect the posterior and anterior pituitary. There is also evidence to suggest selective uptake of cortisol by the neurohypophysis. Thus, the negative feed-back mechanism itself is under neurohypophyseal control. Cytochemical, radioisotopic and electron microscopic studies have further suggested that neurohormones are elaborated in submicroscopic form in secretory neurones the terminals of which abut on neurosecretory cores on pituitary ~~protal~~ portal vessels in the neurohypophysis (Sloper, 1962). Transference from a nervous tissue/

tissue into such vessels must involve the penetration of the so-called "blood-brain barrier". These neurohormones have ACTH-releasing activity and are called corticotrophin - releasing factors (CRF), among which are vasopressin and similar polypeptides, the polypeptide from neurohypophysis, the pepsin - labile extract of the median eminence and other unidentified substances. The neurosecretory process involves the synthesis of the posterior pituitary principle in the neurones of the supraoptic and paraventricular nuclei of the hypothalamus (SON and PVN) and their transfer down the axoplasm of these neurones (the neurosecretory fibres of the median eminence of the upper neurohypophysis) to be stored in, and released from, their terminals in the infundibular process in submicroscopic form (0.1 µ in diameter) which, when aggregated constitutes the microscopic "neurosecretory granules" (1 - 2 µ in diameter). The depletion of the latter granules is a measure of the activity of the hypothalamo - neurohypophyseal system, and their accumulation is a measure of the secretory reserve of this system. Bargmann (1954) was the first to show that such material is also occasionally found near the vascular complexes of the pituitary stalk, an observation which led to the inference that it might be related to the control of anterior pituitary function. Studies have also shown that the tubero-infundibular and other nuclei are very active sites for protein synthesis of these hormones.

So, it seems that the negative feed-back mechanism is a mechanism by which specifically the excessive production of cortisol by the adrenal cortex would tend to inhibit the secretion of ACTH by the anterior pituitary and the diminished production of cortisol to increase the secretion of ACTH. Such a mechanism/

mechanism ultimately depends upon the cortisol content of the pituitary portal blood and the neurosecretory material of hypothalamic origin indicating that this mechanism is under neurohypophyseal and hypothalamic control. Oppenheimer and his colleagues (1961) studied two patients with diencephalic lesions and found that their control levels of urinary steroids were normal, but in neither did the excretion of ketogenic steroids rise when SU-4885 was administered nor were the urinary steroid levels diminished under therapy with dexamethasone, although normal response by adrenals was obtained by stimulation with ACTH. Thus these workers advanced the tentative hypothesis that there existed a 'diencephalic sampling centre' which normally regulated the production of corticosteroids in the body.

The negative feed-back mechanism maintains the constancy of circulating plasma cortisol within relatively narrow limits under normal conditions. Besides its action on the anterior pituitary, there is evidence that cortisol acts also at the hypothalamus and to a very small extent at the adrenocortical level itself (Forsham, 1962).

A second-mechanism comes to action under stressful conditions, either physical or emotional, whereby production of an extra amount of cortisol is ensured to cope with that stressful condition. Thus stressful stimuli act on receptors which initiate impulse conduction in peripheral afferent nerves, somatic and autonomic. Pathways in spinal cord and the cerebral cortex eventually release the sustained inhibition of the reticular formation or the limbic system upon hypothalamic centres in and around the tubero-infundibuler nucleus and the median eminence leading to secretion of CRF by the secretory neurones into the hypothalamo-hypophyseal portal venous system, a link which is sometimes called

"final/

"final common path" (Sayers, 1962). This leads to secretion of ACTH from the anterior pituitary which in turn stimulates the synthesis and secretion of cortisol by the adrenal cortex (figure 3).

Studies of the dynamics of corticotropin activity in man have shown that the initial rate of activation of steroidogenesis is identical over a wide range of doses of ACTH. Thus the adrenals respond with an all-or-none reaction to initial stimulation with corticotropin, or to stress. The duration of the increased secretory activity, however, varies directly with the corticotrophin dose, and thus an optimum output of corticosteroids during the early phases of stress is assured and the duration of increased secretory activity will vary directly with the magnitude of the stimulus. Only at very low doses of ACTH, comparable to the levels found during everyday life, may a graded initial steroidogenic response be demonstrated. Thus a border line response could be obtained by an infusion of 0.25 units of ACTH over 8 hours and a maximum response by infusion of one unit for one day (Bayless and Steinbeck, 1954; DiRaimondo et al, 1955; Beck et al, 1962) denoting that daily output of ACTH is normally less than one international unit. In conditions of stress or after ACTH injection, however, maximum stimulation of both adrenal cortices yields approximately ten times the basal output of cortisol. Use has been made of the inter-relationship between the adrenal cortex and anterior pituitary to establish laboratory tests necessary for diagnostic purposes in various disease conditions. Thus the degree of response of the adrenal cortex to stimulation with exogenous ACTH has been used to test the maximum secretory capacity of the adrenal cortex (Jayle et al, 1959; Beck et al, 1962). This functional reserve capacity of the adrenal cortex can also be tested after strenuous exercise, which is a form of physical stress producing essentially the same effect as ACTH (Diczfalusy/

(Diczfalusy et al,1962). Likewise, administration of synthetic corticoids as alpha-fluoro-hydrocortisone or dexamethasone has been used to test the degree of inhibitory effect of circulating cortisol on the secretion of endogenous ACTH by the anterior pituitary (Liddle, 1960; Liddle and Williams, 1962).

Several other new synthetic drugs such as SU-4885 which blocks 11β-hydroxylation (Liddle et al, 1959; Liddle and Island, 1962) and SU-8000 and SU-9055 which block 17β-hydroxylation (Chart and Sheppard, 1961) could be administered to measure what is called "pituitary-adrenocortical reserve" i.e. the effect of the resulting inhibition of cortisol biosynthesis and release on stimulating ACTH secretion, and accordingly on stimulating other adrenocortical biosynthetic pathways, could be tested, especially as regards congenital deficiencies of adrenocortical enzymes such as in congenital adrenal hyperplasia.

A third and important mechanism controlling the secretion and release of cortisol and other adrenocortical hormones is the musculature and innervation of the adrenal vein previously mentioned. Thick longitudinal muscle bundles are present in the wall of the central adrenal vein, in the centre of the gland the longitudinal muscle is evenly concentric but on either side it becomes typically eccentric. Capillaries or thin-walled venules coming from the cortex enter the lumen of the main vein only between the longitudinal bundles, and never through the thin side of the vein. So, when the eccentric longitudinal muscle in the central vein contracts, the muscle will shorten and its diameter increase to impede the flow from the venules draining the zona reticularis and in view of the innervation previously described Symington (1961) suggested that there is a neuromuscular control which determines the flow of blood from the cortex to the main central vein. Thus from an anatomical and mechanical point of view the function/

function of the longitudinal muscle fibres seems to be one of control of the entering venules whereby they exert an effect on the amount of blood coming from the cortical cells, and accordingly the amount of hormones released into the circulation by the adrenal cortex, as well as the amount of molecular oxygen available to the cells. Since molecular oxygen is required for steroid hydroxylation, Symington (1962) suggested that the adrenal vein musculature, controlled by a neurogenic mechanism, could be one of the controlling factors in steroid biosynthesis and secretion. He also suggested that this peculiar arrangement of the adrenal vein musculature could explain the focal lipid depletion of the adrenal cortex in response to stress.

The physico-chemical state of cortisol in blood

The association between proteins and a large number of substances, including hormones is well known. This association may be close or may be very loose. After being secreted by the adrenal cortex, corticosteroids circulate in the blood stream in varying degrees of "binding" to plasma proteins.

Bischoff and Pilhorn (1948) were the first to suggest that 70-85% of the solubilities of progesterone and testosterone in plasma could be explained on the basis of binding by albumin. West et al (1951) used this hypothesis to explain the limited clearance of testosterone in the kidney, and later on, Eik-Nes et al (1954 b) actually classified a great number of corticosteroids according to their solubility in buffered aqueous and 5% albumin solutions, and concluded that albumin increased the solubility of steroids and that the increase was in inverse proportion to the number of the polar groups (hydroxyl or ketone) in the steroid molecule, while the solubility in aqueous solution is directly proportional to the number of polar groups. These workers suggested that the binding of steroids with serum albumin offers a rational means of transport for the ~~non-benzoid~~ non-benzenoid steroid hormones. Binding of cortisol to plasma proteins, however, was studied by Bush (1957), but it was Daughaday (1958 a, 1958 b) who, by applying electrophoretic studies to plasma with added C^{14}-labelled cortisol actually discovered that, at physiological concentrations in the plasma, cortisol migrated with and was bound firmly to a protein or proteins associated with Cohn fraction IV-4, or more specifically, one of the alpha-one globulins which was called corticosteroid-binding-globulin (CBG). At higher plasma cortisol concentrations (above 30 µg./100ml.) the binding/

binding protein becomes saturated and the additional cortisol is loosely bound by albumin. The capacity of albumin to bind cortisol is relatively large. Cortisol binding by alpha globulin was soon confirmed (Slaunwhite and Sandberg, 1959; Sandberg and Slaunwhite, 1959) but the protein was given a different name; "transcortin".

Steroid binding by CBG is made stronger by inclusion of hydroxyl groups at the 11β, 17α and 21-positions of the molecule. Thus cortisol is firmly bound to CBG with corticosterone competing for the same binding site while aldosterone is not bound to CBG but chiefly and minimally to albumin. Definite selectivity of the steroid interactions with the different plasma proteins was shown by Westphal (1957), who showed also that plasma proteins bind steroids through their Δ^4-3α ketogroup. The chemical nature of this interaction is unknown; polar interactions and van der Waals forces have been suggested. Thyroxine-protein linkage is strong enough to withstand precipitation of the proteins. Nevertheless butanol will extract the thyroxine from plasma; this is effected by solubility and not by cleavage of a chemical link. Steroids are even more loosely bound to protein and any organic solvent which will denature the protein will extract steroids from plasma.

Cortisol is 90-95% protein-bound in the plasma at physiological levels (Mills et al, 1960) and the degree of binding is inversely related to temperature; at 37°C, 6-7% of cortisol is unbound but at 4°C only 2% is unbound. On the other hand the degree of steroid binding is not highly dependent on pH (Chen et al, 1961).

In its function as a carrier of cortisol in the plasma, the CBG mechanism assures a ready source of available circulating hormone, protecting it from conjugation in the liver and subsequent excretion in urine, but at the same time limitation of diffusion into tissues must ensue (Daughaday, 1958 a, 1958 b; Sandberg/

Sandberg and Slaunwhite, 1959; Slaunwhite and Sandberg, 1959; Peterson, 1960). These authors, and Wallace and Carter (1960), suggested that protein-bound cortisol would not be available for direct metabolism by the usual routes and that only the non-protein-bound fraction is the physiologically active moiety at the tissue or cell level.

When binding is raised by estrogen therapy the half-time for disappearance was found to be increased from 60 to 120 minutes as estimated using C^{14}-labelled cortisol (Mills et al, 1960). Mills et al (1960) and Peterson (1959 a) showed that estrogen treatment does not alter the biologically active fraction of plasma cortisol as measured by the non-protein-bound fraction, and concluded that this binding globulin will, therefore, provide a buffer action against rapid changes in the biologically active fraction of plasma cortisol. Thus it would take up cortisol if secretions were suddenly increased or provide a reserve for a short time if the secretion rate falls. This view seems to be universally accepted nowadays and it explains the low titres of CBG in cerebrospinal and amniotic fluids and also explains the presence of great amounts of unmetabolised cortisol in urine, in the presence of great amounts of unbound cortisol in the blood, as a result of more effective glomerular filtration. Conjugated steroids are highly water-soluble, do not bind at all and are readily filtrable through the kidney.

Production of CBG occurs chiefly in the liver but the mode of its synthesis and metabolism are still unknown. Seal and Doe (1961) claimed to have isolated the cortisol-binding-globulin; they stated that it is a glycoprotein;/

glycoprotein; the protein contained 0.3% fucose (methylpentose), 2.7% sialic acid, 3.6% hexosamine and 3.8% hexose. Normally the mean CBG binding capacity of human plasma studied with a double equilibrium dialysis method using 4-C^{14}-cortisol, is about 20.0 ± 5.0 μg. cortisol/100ml. (Daughaday et al, 1962), the steroid-binding capacity of plasma being defined as the amount of steroid which can be held by protein in the plasma, and is dependent upon the concentration of protein in the plasma and upon the number of molecules of steroid bound to each molecule. DeMoor and Meulepas (1962) showed that the critical level above which disposal of cortisol is no longer influenced by transcortin binding is 25-30 μg./100ml.

The production of CBG is markedly increased in pregnancy and after estrogen treatment (Slaunwhite and Sandberg, 1959; Sandberg and Slaunwhite, 1959; Mills et al, 1960) without clinical manifestation of hyperadrenocorticism since most of the hormone is bound and thus not available to the tissues. Conversely a lowering of CBG activity is noted in the newborn and in various clinical abnormalities of serum proteins i.e. dysproteinaemias, e.g. liver cirrhosis, nephrosis, multiple myeloma, exudative enteropathy, etc. (Sandberg and Slaunwhite, 1959). No diurnal variation of CBG titre occurs, nor is it affected by corticotrophin (Mills, 1961 b).

Metabolism of cortisol

The main site of cortisol metabolism is the liver which is able to convert, within two hours, about two-thirds of a labelled cortisol dose into the conjugated form.

Practically no destruction of the steroid ring occurs in the metabolism of cortisol. The main metabolic pathways of cortisol are illustrated in figure 1. The first step in cortisol metabolism is the reduction of the double bond between C-4 and C-5, by the incorporation of two hydrogen atoms in ring A, catalysed by the TPNH-dependent enzyme Δ^4-hydrogenase, to form the biologically inactive compound dihydrocortisol (5β) and its allo-derivative (5α). Next, the α-3 ketonic group is reduced, by the incorporation of another two hydrogen atoms, and tetrahydrocortisol (Lieberman and Teich, 1953; Roberts and Szego, 1955; Tomkins, 1956) and allo-tetrahydrocortisol (Bush and Willoughbey, 1957) are formed. This step is catalysed by the enzyme 3α-hydroxysteroid dehydrogenase, TPNH or DPNH dependent. But as cortisol and cortisone are interconvertible *in vivo* (Lieberman et al, 1953; Gray and Lunnon, 1956; Bush, 1956; Peterson et al, 1957 b; Bush and Mahesh, 1958; Bush and Mahesh, 1959 a, 1959 b) dihydrocortisone, allo-dihydrocortisone, tetrahydrocortisone and allo-tetrahydrocortisone will be similarly formed during cortisol metabolism (Roberts and Szego, 1955; Bush and Mahesh, 1959 b). The rate limiting step is the first reduction to dihydrocortisol. Biological activity of cortisol is lost when the Δ^4-3α-ketone group is reduced. Mostly, these tetrahydro derivatives are conjugated through their 3α-hydroxyl groups, by the action of the enzyme glucuronosyl transferase, with uridine diphosphate glucuronic acid (glucosiduronic acid) to form the corresponding tetrahydro/

tetrahydro-3α, β-d-glucosiduronate, or simply glucuronides (Isselbacher, 1956), and to a much lesser extent conjugated with sulphuric or phosphoric acid to form the corresponding sulphates or phosphates. Glucuronides circulate in the blood unbound to protein. They are readily water-soluble and are rapidly excreted in the urine without any significant reabsorption by the tubules.

A further step in cortisol metabolism is the reduction of the carbonyl group at C-20, where two more hydrogen atoms are incorporated, to give secondary alcohols. The resulting compounds derived from cortisol and cortisone are called cortols and cortolones respectively. These can be α- or β-cortols and cortolones depending onthe position of the hydroxyl group at C-20, all of which have β-hydrogen at C-5. Allo-cortols and allo-cortolones are also found which have an α-hydrogen at C-5 (Fukushima et al, 1955; Fukushima et al, 1960). A last, and quantitatively unimportant step in cortisol metabolism involves the loss of the side chain of cortisol, cortisone, their dihydro- and tetrahydro-derivatives and of cortol and cortolone and its replacement by a ketone group; i.e. the formation of 17-ketosteroids; 5-8% of isotopically-labelled cortisol appears in this form in urine (Sandberg et al, 1957 a; Edwards, 1961). These comprise; 11-keto and 11-β hydroxy-etiocholanolone and the corresponding androstane derivatives; 11-keto- and 11β-hydroxy-androsterone.

A further smaller proportion of cortisol is excreted unchanged in the urine either unconjugated (Peterson et al, 1955; Neher, 1959) or partly conjugated, at C-21 to sulphuric acid or glucosiduronic acid (Pasqualini, 1960; Pasqualini and Jayle, 1961). It was noticed that with increased plasma concentrations of cortisol, a disproportionate increase in the urinary excretion/

excretion of the primary cortisol occurs (Greaves and West, 1960; Kornel and Hill, 1961) probably as a result of the increased level of the unbound plasma cortisol being readily filtrable through the glomeruli of the kidney.

20-40% of intravenously administered C^{14}-labelled cortisol appears in the urine as the tetrahydro-derivatives; THE, THF, allo-THE, 20-30% appears as cortol and cortolone, 7% as the 17-ketosteroids; 11-keto and 11β-hydroxy-etiocholanolone (5%), 11β-hydroxyandrosterone (2%) and 11-keto-androsterone (less than 1%) (Sandberg et al, 1957 a; Gallagher, 1959; Fukushima et al, 1960) and only very small proportion of the hormone is excreted unchanged. THE is the main metabolite and it constitutes roughly about two-thirds of all recognisable urinary metabolites of cortisol.

The ratio of 11-hydroxy to 11-keto tetrahydro-derivatives is normally constant. This, in practice is represented by the ratio of THF to THE or THF plus allo-THF to THE and lies in the range 0.8 - 1.7 (Bassöe et al, 1958; Romanoff et al, 1958; Hellman et al, 1961; Maeyer et al, 1963). These workers also found that the 5α-metabolite allo-THF comprises 9-25% of all four tetrahydro-derivatives. The ratio of 11-hydroxy to 11-keto tetrahydro-derivatives tends to increase with increased total excretion due to increased secretion or to administration of exogenous cortisol (Gold et al, 1958; Gold et al, 1959; Gray et al, 1962; Cost, 1963). Romanoff and his associates (1961) using C^{14}-labelled cortisol found that the ratio of the two main 11-hydroxyl metabolites of cortisol THF and allo-THF, to the two main 11-ketone ones, THE and cortolone, is constant in relation to age, and so was also the proportion of the one 5α-metabolite allo-THF to the three main 5β-metabolites THF, THE and cortolone.

Cleavage/

Cleavage or destruction of the steroid nucleus *in vivo* and excretion of cortisol or its metabolites by extrarenal channels are quantitatively unimportant routes of its disposal by man (Hellman et al, 1954).

The half life of free cortisol in the plasma, as estimated by isotopic cortisol is normally between 60 and 120 minutes. As the liver is the main site of metabolism of cortisol, the half life is usually prolonged in the presence of liver dysfunction and is reduced with conditions of increased metabolism, e.g. in thyrotoxicosis. The rate of metabolism of cortisol can be judged by the fact that about 60% of a tracer dose of cortisol will be metabolised and excreted in urine in a period of six hours, about 80% of that dose will be metabolised and excreted in urine in 24 hours, 90% in 48 hours, and about 95% in 72 hours (Hellman et al, 1954; Romanoff et al, 1961; Flood et al, 1961; Layne et al, 1962). Only about 5% of radioactivity has been found in the faeces in the first 48 hours and less than 0.5% was reported to be present as CO_2 in expired air which indicate that excretion in faeces and degradation of ring A represent only a minor pathway and that the main excretion occurs in fact in urine. Hellman and his co-workers (1954) suggested that these figures indicate that the rate of metabolism of cortisol is independent of tissue requirements and of the amount present in the body and, furthermore, since a small percentage is excreted as unaltered cortisol, the rate of metabolism may be inferred to be rapid.

Recently, however, some alternative pathways for cortisol metabolism were suggested; Bradlow et al (1962) using isotopic studies proved that some of the metabolic products formed *in vivo* from cortisol could well be derived through a path that has a reduction of the C-20 ketone as the first stage, and accordingly,/

accordingly, that a portion of the other metabolites including the cortols, cortolones and 11-keto and 11-hydroxy- 17-ketosteroids may be derived from an intermediate C-20-hydroxy compound. They stressed, however, that this is not a major route of cortisol metabolism.

Furthermore, the identification and isolation of 6β-hydroxy-cortisol by Burstein and his co-workers in 1954 led to extensive research to study this compound in relation to cortisol metabolism. Touchstone and his associates (1959) suggested that 6β-hydroxycortisol is secreted independently from the adrenal cortex, but Ulstorm and his co-workers (1960) injecting C^{14}-labelled cortisol to infants in the first postnatal week showed that 6β-hydroxy-cortisol, a relatively polar metabolite, is excreted in larger amounts than in adults, and concluded that very polar metabolites of cortisol are more important pathways of cortisol disposal in newborn infants than they are later in life and, accordingly, that degradation pathways of minor quantitative importance in adults are more extensively used during the postnatal period. That 6β-hydroxy-cortisol is a metabolite of endogenous cortisol rather than an independent secretion from the adrenal gland and in fact that it is the most abundant unconjugated corticosteroid in normal human urine was confirmed by other workers using also C^{14}-labelled cortisol (Cohn et al, 1961; Lipman et al, 1961; Lipman et al, 1962, Katz et al, 1962). Frantz and his associates (1960) noticed the increased excretion of this compound in urine of pregnant women and stressed the importance of this metabolic pathway of cortisol in pregnancy. Furthermore, by studying the excretion of this compound after estrogen treatment, it was suggested that 6β-hydroxylation is an alternative route of cortisol metabolism which may be of quantitative importance in pregnant or estrogen treated subjects since the excretion of conjugated corticosteroids has been found to be diminished in similarly treated subjects (Lipman

et/

et al, 1961; Lipman et al, 1962). Increased excretion of this compound was also noticed in cases of adrenocortical hyperfunction (Touchstone and Blakemore, 1961; Frantz et al, 1961). 6β-hydroxylation occurs mainly in the liver and hydroxylation of cortisol at various carbon atoms is known to render cortisol more water soluble and capable of being excreted in the urine in the unconjugated state (Lipman et al, 1962). Excretion of 6β-hydroxycortisol and probably of other highly oxygenated corticosteroids assumes a more important role in certain states such as pregnancy, exogenous hyperestrogenism, liver disease, adrenocortical hyperfunction, and the newborn period (Lipman et al, 1962; Katz et al, 1962). Trying to find the relationship between these conditions and the excretion of 6β-hydroxycortisol Katz and his co-workers (1962) suggested that the utilization of this alternate pathway of cortisol metabolism may be increased when reduction of ring A is impaired. This was, however, suggested before by Bush and Mahesh (1959 b) who noticed that hydroxylation of corticosteroids became much more important with steroids in which the major reductions in the A ring and at C-20 are partially or totally blocked, and as it has been shown that estrogen treatment leads to decreased formation of TPNH which is necessary for reduction of ring A (Peterson et al, 1960), so the resulting reduced formation of THE and THF may favour the formation of 6β-hydroxycortisol. Thus, increased excretion of 6β-hydroxycortisol in exogenous hyperestrogenism is explained, and its increase in pregnancy could similarly be due to the increase of circulating level of estrogen. Moreover the preponderance of unconjugated polar steroids in neonatal urine may reflect the effect of high maternal estrogen on cortisol metabolism in the newborn; large urinary excretion of estrogen metabolites have been reported to occur during/

during the first few days of life (Diczfalusy et al, 1957).

Estrogen also can account for the somewhat higher levels of 6β-hydroxycortisol in normal females than in males. In liver disease the elevated excretion of 6β-hydroxycortisol may be due to the relative inability of the diseased liver cells to reduce ring A. But increased excretion of 6β-hydroxycortisol in adrenocortical hyperfunction could be due to the saturation of the enzyme systems of the major pathways by the abnormally increased quantities of cortisol to be metabolised. Thus it seems that the formation of 6β-hydroxycortisol and other polar metabolites of cortisol presents a metabolic "run off" in situations in which the pathways for the formation of the more common derivatives of cortisol are either impaired or saturated.

The other polar metabolites of cortisol identified and isolated are 20-hydroxy-6β-hydroxycortisol (Lipman et al, 1961; Katz et al, 1962) 6β-hydroxycortisone (Lipman et al, 1961; Katz et al, 1962; Pasqualini et al, 1963), 2α-hydroxycortisone (Lipman et al, 1961), 6α-hydroxycortisol (Frantz et al, 1961) and other unidentified 20,21-ketols (Katz et al, 1962). Bush (1962) summarises the metabolic pathways for corticosteroid metabolism as:-

(1) Hydrogenation of the 4,5-double bond to give either 5β- or 5α-hydrogen atom.

(2) Oxidation-reduction of secondary hydroxyl and carbonyl groups; at positions 3, 11 and 20 predominantly in the reductive direction, and at position 17 a considerable amount of the ketone results.

(3) Hydroxylation at positions 2,6, 16 and possibly at other positions.

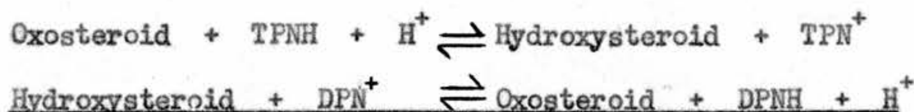
(4) Conjugation of hydroxyl groups either as ester sulphates or as glucuronosides, and probably with other radicals.

Mode of action of cortisol

The mode of action of cortisol, and other corticosteroids, is not yet fully understood. Pincus (1962) justifiably states that the metabolic transformations which these secreted hormones undergo are probably not indicative of their modes of action, and that the surprising pyrogenic action of etiocholanolone and the hypocholesteremic action of androsterone should make us think twice before we dispose of steroid metabolites as mere excretion products. He goes on to discuss the various hypotheses put forward to explain the mode of action of cortisol, viz:- (1) corticosteroids act as co-factors or catalytic active centres in a nucleotide or protein complex, (2) corticosteroids act as intervenors in a transport system operating at the cell surface or at the surface of intracellular enzyme sites, (3) corticosteroids act to "activate" specific enzyme systems by any of several means such as competitive action against a bound inhibitor or the disruption of protein linkages which need to be 'free' for functional activity. He also suggests other possible roles of steroids as (1) inducers of specific enzyme synthesis in target sites; examples are seen in the rapid increase in β-glucuronidase concentrations under estrogen stimulation or of uterine carbonic anhydrase concentrations by progestins, and (2) causes of enzyme adaptation to their presence, which means that corticosteroids may induce the intracellular formation of proteins with high binding affinities for the specific molecular configurations presented by each of the hormonal steroids.

Talalay (1962) reviewed the first hypothesis, proposed earlier by his group of workers (Hurlock and Talalay, 1958), which stated that certain specific hydroxysteroid dehydrogenases which reacted with both DPN and TPN could promote a reversible transfer of hydrogen between the oxidised and reduced forms/

forms of these pyridine nucleotides in the presence of catalytic concentrations of the appropriate steroid substrates, and that during these transhydrogenation reactions, the steroids undergo reversible oxidation-reductions and function catalytically as hydrogen carriers or coenzymes. This could be represented by:



$$\text{Oxosteroid} + \text{TPNH} + H^{+} \rightleftharpoons \text{Hydroxysteroid} + \text{TPN}^{+}$$
$$\text{Hydroxysteroid} + \text{DPN}^{+} \rightleftharpoons \text{Oxosteroid} + \text{DPNH} + H^{+}$$

$$\text{Sum:} \quad \text{TPNH} + \text{DPN}^{+} \rightleftharpoons \text{DPNH} + \text{TPN}^{+}$$

Talalay, thus suggested that some manifestations of steroid hormone action might involve regulation of intracellular hydrogen balances. This, he stated, involves interactions of the steroids with enzymes some of which at least exhibit the affinity and structural specificity which are required of the complementary cellular receptors for steroids. He concluded that, although there is little direct experimental evidence for this view, if steroid-mediated hydrogen transport represents one aspect of the mechanism of steroid hormone action, this would provide a functional role for some of the known metabolic transformations of steroids and, moreover, that such a process would reconcile the mechanism of actions of the steroids with those of other steroid substances which may function in a catalytic role as coenzymes.

Ingle (1962) stated that the presence of hydroxysteroid dehydrogenases with steroid specificity and dual pyridine nucleotide specificity, such as the 11β-hydroxysteroid dehydrogenase which interconverts cortisone and cortisol with the participation of either pyridine nucleotide as hydrogen carrier, has been demonstrated, and that as the balance between pyridine nucleotides plays an important role in regulating the balance between various synthetic and energy-yielding reactions, he supports Talalay's hypothesis that corticosteroids act as coenzymes/

coenzymes and that the 'action' and 'metabolism' of steroid hormones may be part of the same process; as the steroids regulate intracellular hydrogen traffic, they undergo reversible metabolic changes. However, he admitted that a lot of information is still missing, such as the need for demonstration of deficient intracellular hydrogen traffic in adrenalectomised and castrate animals, the answer to the question of whether this reaction is only catalysed by each 'active' steroid and whether non-steroid electron carriers without hormonal activity can act in this system.

These questions were partly answered by Bush (1962) in an excellent article, in which first of all he rejected the idea that corticosteroids act as coenzymes or catalytic substances in view of the fact that the metabolic reactions undergone by the steroid hormones are now largely thought of as steps in their inactivation and elimination from the organism. He also rejected the theory of the possible action of corticosteroids as activators of specific enzyme systems in view of the fact that no specific physiological effect of a steroid hormone has yet been shown to be due to a 'direct' action upon an enzyme or enzyme system. He also rejected the proposal that the mechanism of action of corticosteroids is to form chelates with important metal ions in view of the fact that it is known now that the α-ketol side chain is not essential for sodium-retaining activity (Fried and Borman, 1958). He than studied the effect of structural modification of the steroid molecule on the spatial configuration, molecular volume and distribution of electrons in the molecule and showed that many α-sided substituents would block oxidation-reduction at C-11 whereas they enhanced the biological activity of analogues containing the 11β-hydroxyl group and that only the 11β-ol can combine with

and/

and directly affect the receptor. Hydrogen of the 11-β-hydroxyl group takes part in a hydrogen bond with a proton-accepting group in the receptor. In the natural steroids this bond is too weak to produce much activity unless the side-chain contains both the 20-ketone and the 21-hydroxyl groups for attachment to the receptor by semi-polar or hydrogen bonds. About two thirds of cortisol metabolites are 11-ketones, which means that the oxidation of the 11-β hydroxyl group is probably an important 'wastage' reaction. This is because every molecule of 11-ketone that is produced extrahepatically can only be reduced to the active 11β-ol by passage through the liver and this passage is attended by the loss of about 50% of the ketone through the usual metabolic reactions in ring A and the side-chain. Applying this to studies using 2α-methylcortisol, Bush (1962) suggested that α-sided substituents will protect the 11β-hydroxyl group from oxidation as well as blocking the usual inactivating reactions in ring A and/or the side-chain. These effects became particularly apparent with 9α- and 12α-halogeno-steroids; the negative inductive effect of the halogen increased the acidity and hence the hydrogen-donating tendency of the 11β-hydroxyl group; hydrogen bonding to a proton-accepting group on the receptor would therefore be strengthened. Bush showed that numerous analogues of glucocorticoids, often far more active than the natural corticosteroids, exist in which the α-side of the molecule is grossly deformed by large substituents or by angulation of the A/B-ring junction and suggested that it is very unlikely that the steroid is attached to any part of the natural receptor for these substances by its α-side. On the other hand large substituents axially substituted on the β-side of the molecule reduce glucocorticoid activity to vanishing point. Therefore, it/

it seems likely that the natural receptors for glucocorticoids combine closely with the steroid by the β-surface over the regions of the side-chain and at least the C and D rings, and that the 11β-hydroxyl group is probably one of the main sources of specificity in the attachment of glucocorticoids to their receptors. Bush then concluded that the steroid hormones produce their direct effects by some simple consequences of association with specific receptors in the target tissues and that this association appears not to involve any metabolic reaction in which covalent bonds are broken. He also concluded that both the association and the direct effect ensuing from it are very sensitive to the shape and size of certain regions of the steroid hydrocarbon skeleton, and to the position and orientation of one special polar group. In the natural steroid hormones, less specific interaction with one or two other polar groups may aid their interaction with their receptors but both the steric and electronic requirements are less strict than for the former type of polar group, and the groups themselves can be dispensed with entirely in suitably modified analogues. Bush also concluded that in glucocorticoids the fit with the receptors is likely to be around the β- and 'top' - sides of the C and D rings and very loose contact, if any at all, between the 'bottom' edge of the steroid molecule and its receptor; little or no close fit occurs with the receptors around the α-side of the molecule especially at the C and D rings; and an interaction of the A- and B-ring structures could only occur with a part of the receptor that is flexible or else be non-existent. He admitted, however, that the nature of the direct effect of these hormones is still unknown at present, but he stated that whatever the nature of the receptors for steroid hormones, a large area of contact by rather weak bonds must be postulated to account for the specificity of the steroid-receptor combination,/

combination, and further, that in the absence of reactions breaking covalent bonds, and the absence of reactive parts of the molecule likely to undergo presently undiscovered reactions, the only 'event' that can at present be postulated to take place during the association of the steroid with its receptor is the combination itself. The steroid as it were, 'sits down' on the receptor in a specific orientation and the job is done, and that the two most likely consequences of such a molecular 'event' are that the conformation of the receptor molecule is altered, or that the access of another type of molecule to that region of the receptor is prevented by the presence of the steroid, and that the biochemical manifestations of this type of event might be in the field of enzyme activity or the 'permeability' of cellular or intracellular membranes and remains to be discovered.

Metabolic effects of cortisol

Although it is a common belief that individual corticosteroids have either an inorganic effect, such as that of mineralocorticoids on sodium retention or potassium excretion, or an organic effect such as that of glucocorticoids, androgens, estrogens or progesteroids on protein anabolism or catabolism, yet there is a considerable functional overlap between the two groups; cortisol for example, a glucorticoid, has a significant effect on electrolyte metabolism, while aldosterone, the principal mineralocorticoid, has five hundred times the activity of cortisol in sodium retention yet only one-third its gluconeogenic potency.

The different metabolic effects of cortisol and other corticosteroids were reviewed by Forsham (1962). The principal metabolic effect of glucocorticoids in general and of cortisol in particular is directed towards carbohydrate metabolism. They cause marked enhancement of gluconeogenesis (Long et al, 1940), which leads to very large increases in liver glycogen, catabolism of tissue proteins with marked nitrogen loss, hyperglycaemia and glycosuria. The latter two effects of cortisol are due largely either to direct augmentation of gluconeogenesis or to a reduction in utilization of glucose by the cells, or both (Long and Smith, 1962) and to a very small extent to its peripheral antagonistic action to insulin in conjunction with growth hormone. The rise in blood sugar is temporarily opposed by increased secretion of insulin, but eventually diabetes, called 'steroid diabetes', may follow. Steroid diabetes is seldom associated with ketoacidosis. At the physiological level, cortisol effect on fat metabolism induces centripetal redistribution of fat, meantime increasing the total amount at the expense of protein. Chronic excess of cortisol/

cortisol leads to increased lipolysis, hyperlipaemia, hypercholesterolaemia, ketogenesis and redistribution of triglycerides in fat depots producing the so called "spider appearance" characteristic of Cushing's syndrome.

Cortisol has a definite effect on water metabolism, viz. enhancement of water diuresis. In absence of cortisol, a water load is not disposed of for twelve hours or more, a fact which forms the basis of the so-called water test of adrenal cortical insufficiency (Moses et al, 1958). Cortisol also prevents the passage of water into the cell, thus maintaining the extracellular fluid volume, which, in turn, preserves adequate glomerular filtration and ensures water diuresis. This seems to be a more accepted hypothesis than that assuming direct antagonistic effect of cortisol on the pituitary antidiuretic hormone at the kidney tubules or enhancing its destruction in the liver. There is no evidence to support the hypothesis that cortisol suppresses the secretion of antidiuretic hormone at the hypothalamic-pituitary level.

Cortisol and other glucocorticoids, with or without a 17-hydroxyl group, lyse fixed lymphoid tissue and reduce the number of circulating lymphocytes. Glucocorticoids, with a 17-hydroxyl group, depress the number of circulating eosinophils; this effect is not dependent upon suppression of bone marrow production but to increased sequestration of circulating eosinophils in the lungs and spleen and their destruction in the circulation. Basophils suffer a similar fate. On the other hand the relative and absolute neutrophil count is raised, so that the overall picture in cortisol excess is an increased total leucocytic count, relative and absolute neutrocytosis, lymphopenia and eosinopenia. The phagocytic system of the blood is neither suppressed nor stimulated. Erythrocyte and thrombocyte counts are also increased leading to polycythaemia and thrombocytosis.

Cortisol/

Cortisol also affects striated muscles; in the absence of cortisol, muscle weakness occurs which can not be improved by administration of deoxycorticosterone, saline or glucose. Cortisol excess also leads to muscle weakness due to depletion of muscle protein, oedema, fibrosis and probably also due to potassium depletion.

Cortisol also has an effect on the central nervous system. In deficiency states, slowing of the electrical discharges in the electroencephalogram occurs. On the other hand, in cortisol excess, the threshold for electrical excitation of the brain is lowered, accounting for an increased tendency to epileptic seizures. Psychiatric disturbances are also common both with lack and excess of cortisol; as many as 40% of patients with Cushing's syndrome show some major psychiatric disturbance and in fact most of the early cases of that syndrome described by Cushing were patients of mental hospitals.

On the gastrointestinal tract, cortisol induces increased gastric acidity and to a lesser extent pepsin production, thus accounting for the well recognised tendency to peptic ulceration in patients with excess endogenous cortisol secretion (Grey et al, 1953). These effects can be blocked by anticholinergic drugs.

The effect of cortisol on bone metabolism is towards enhancement of every phase of osteoporosis. Thus in excess, cortisol inhibits the synthesis of the organic matrix, by impeding the development of cartilage and accordingly thinning of epiphysial plate and interruption of growth of bone; the formation of new bone is blocked at the level of the matrix and osteoblasts are scarce. Cortisol also causes decreased deposition of calcium and increases the renal clearance and urinary loss of calcium. These effects are aggravated by cortisol antagonising the action of vitamin D which leads to decreased absorption of calcium from the gut.

The most important effect of cortisol on the cardiovascular system is the sensitisation of the arterioles to the pressor effect of norepinephrine. Cortisol, however,/

however, may per se raise the blood pressure to some extent. It enhances the production of angiotensinogen, resulting eventually in higher levels of angiotensin which stimulates aldosterone production which in turn further increases the blood pressure (Forsham, 1962). By its mineralocorticoid effect, cortisol participates in maintaining adequate sodium/serum levels and circulating blood volume. Excess cortisol, however, leads to a tendency to atherosclerosis following a rise in circulating blood lipids and cholesterol levels. This may be due to the effect of cortisol on cholesterol, decreasing its diffusion into the vessel wall.

The effects of cortisol and related 17-hydroxylated derivatives on the mesenchymal tissues are of greatest importance. Normally, as a result of an injury or a focus of infection, in the acute phase, perivascular exudation and marked diapedesis occur and neutrophils surround the site of infection. In the subacute stage lymphocytes predominate and in the chronic stage of healing fibroblasts surround the focus, walling it off. Subsequently the blood vessels become narrowed by dense fibrosis. In the presence of excess cortisol, there is a decrease in hyperaemia, less exudation, diminished diapedesis, cellular migration and infiltration. The major tissue response is one of mononuclear cells. The early phase of repair is retarded by inhibition of fibroplasia and late fibrosis.

Cortisol also interferes with intracellular synthesis of histamine without antagonising its action; thus it will not inhibit the local response to histamine and will only affect an acute allergic response a few hours later when the histamine present is destroyed and its inhibiting effect upon further production of histamine becomes manifest.

Antibody/

Antibody changes may also occur with large doses of cortisol where initial liberation of excess antibody occurs subsequently followed by fall due to suppression of antibody formation. Cortisol inhibits hypersensitivity reactions to antigen-antibody complexes, probably by interposing a protective layer between the complex and the target cell surface. The inhibition of production and the subsequent reduction of the circulating levels of antibodies by very large doses of cortisol is due to lysing fixed plasma cells and lymphocytes. The resistance to infection, already diminished by the mesenchymal effects of small doses is further aggravated by doses large enough to reduce antibody formation.

The metabolic effects of mineralocorticoids are mainly directed towards control of sodium and potassium levels and water volume in intracellular and extracellular fluids mainly by their action at the distal tubules of the kidney. The same action occurs to a smaller extent in the salivary glands, sweat glands, intestinal mucosa and in exchanges between extra- and intracellular fluid throughout the body.

It must be pointed out that the amounts of cortisol needed for all the metabolic effects mentioned to sustain life and homeostasis are greatly increased by physical or emotional stress and, on the other hand, amounts needed to keep the animal living under extreme stress will cause signs of hypercorticolism in the resting state (Ingle, 1962).

In addition to these "specific actions" on the various systems of the body, adrenal cortical hormones especially glucocorticoids are known now to exhibit a supportive or 'permissive' action (Ingle, 1954) whereby other hormones exert their effect only in the presence of small amounts of glucocorticoids. Ingle defined the conditions implied in this theory:

1./

1. The metabolic response to a stimulus occurs in the presence of the adrenal gland.

2. The response is not demonstrable when the gland is removed and no replacement therapy is given.

3. The response is again elicited when a steady intake of cortisol hormones is substituted for the adrenal gland.

So, under normal conditions, the eucortical state is necessary to support biological responsiveness but the cause which incites the response is of extra-adrenal origin. This view was supported by Crane (1962) who showed that many of the metabolic and pharmacological responses to stress can develop fully in the absence of the adrenal glands when small constant doses of adrenocortical hormones are given. Under such circumstances, the adrenocortical hormones permit or 'normalise' the responsiveness of extra-adrenal mechanisms. Thus the metabolic effects of cortisol assumes that the primary actions of cortisol and other corticosteroids are necessary to maintain the normal functions of cells and entire organs and their responsiveness to extra-adrenal stimuli especially so under conditions of exposure to severe stress (Ingle, 1962; Crane, 1962). An example of this action is the release of significant amounts of glucose from liver glycogen by epinephrine and glucagon only in the presence of cortisol. This permissive action also serves to explain the occasional restoration of blood pressure by cortisol in patients in shock who no longer respond to norepinephrine alone. Thus the empirical treatment of different cancers by bilateral adrenalectomy may depend on the carcinogenic process proceeding at suboptimal speed in the absence of the permissive effect of corticosteroids./

corticosteroids. Likewise the relationship of corticosteroids to diseases such as diabetes and hypertension may be permissive rather than causal (Ingle, 1962).

Crane (1962) concluded, however, that the term 'permissive' described this inter-relationship of the corticosteroids and certain processes of metabolism and disease but does not define the ultimate mechanisms involved; the fundamental biophysical and biochemical processes in which the adrenal cortical hormones participate at a cellular and molecular level are still largely unknown.

Physiological variations of adrenocortical activity

Variation of adrenocortical activity as regards sex, could not be demonstrated in the plasma level of cortisol (Bliss et al, 1953; Lewis, 1957; DeMoor et al, 1960 b) although some data suggest that values in women may be lower than in man (Bondy and Upton, 1957). Urinary 17-ketosteroids and 17-hydroxycorticosteroids are, however, usually higher in males than in females (Norymberski et al, 1953; Reddy, 1954; Levell et al, 1957; Borth et al, 1957; Loraine, 1958).

This difference has been attributed by some workers to the difference in weight of total muscle mass between the two sexes, which implies that the difference will disappear if corticosteroid excretion is correlated to creatinine excretion or body weight (Romanoff et al, 1961; Mlynaryk et al, 1962) and a better way of estimation of urinary corticosteroids seems to be in expressing the results as mg./Kg. body weight/day.

Spontaneous diurnal variation, sometimes called 'circadian rhythm', of a constant pattern occurs normally in persons not subjected to stress. This was first described by Pincus (1943 a) in terms of 17-ketosteroid excretion. But a diurnal variation in the level of 17-hydroxycorticosteroids in plasma (Bliss et al, 1953; Gemzell, 1953; Sandberg et al, 1954; Tyler et al, 1954; Doe et al, 1956; Migeon et al, 1956 b; Brown et al, 1957; Doe et al, 1960 a) and urine (Doe et al, 1954; Laidlow et al, 1954; Doe et al, 1960 a) is more pronounced. This has been confirmed by a more significant method for cortisol estimation in plasma (Lewis, 1957). Plasma 17-hydroxycorticosteroids is highest between 6:00 and 8:00 a.m. falling to a minimum around midnight (Bliss et al, 1953; Sandberg et al, 1954; Migeon et al, 1956 b; Doe et al, 1956; Brown et al, 1957;

Perkoff/

Perkoff et al, 1959), but considerable variation exists between subjects, both with regards to the peak time and the degree of fluctuation observed. No sex difference of this circadian rhythm has been reported. Urinary changes are essentially the same although there may be a short lag between them and those of the blood. About 70% of the 17-hydroxycorticosteroids secretion by the adrenal cortex occurs between midnight and 6:00 a.m., and the difference between maximal and minimal secretory activity, reflected as plasma 17-hydroxycorticosteroid level, is about two-fold in most cases. This cycle is independent of exercise or food intake. Night workers and blind persons have normal rhythms (Tyler et al, 1954; Migeon et al, 1956 b) but significant loss of diurnal rhythm occurs in clinical conditions associated with alterations in consciousness or sleep patterns, and total reversal of sleep and activity for five to ten days was reported to result in a reversal of the normal rhythm (Perkoff et al, 1959). It was also noticed that adaptation of the ketogenic steroid excretory rhythm to a reversed activity-sleep and light-darkness schedule, in places where day light persists for twenty-four hours, occurs in two to eight days (Sharp et al, 1961) denoting that these rhythms probably depend upon habit and environment and synchronization of pituitary and adrenal responsiveness.

Eik-Nes and Clark (1958), however, noted the lack of diurnal variation in four patients following severe head injury. This has led other workers to try to localise specific areas of the brain as centres for this diurnal variation. Hökfelt and Luft (1959) noted the absence of normal variation in free plasma cortisol in patients with suprasellar extension of pituitary tumours, implying the role of hypothalamic control of ACTH secretion and diurnal rhythm. Krieger (1961) noticed abnormal patterns of 17-hydroxycorticosteroids in plasma of patients suffering from pretectal and temporal lobe lesions of the brain and concluded/

concluded that these areas of the brain are important centres in regulation of pituitary release of ACTH and of the diurnal pattern of plasma 17-hydroxy-corticosteroids which is a reflection of such a release. Oppenheimer and his colleagues (1961), however, showed that the mechanisms leading to the normal diurnal variation of plasma 17-hydroxycorticosteroids and to the normal adrenal response to stressful stimuli do not involve those diencephalic centres postulated to control the reciprocal pituitary-adrenal inter-relationship.

Most probably, however, this cycle represents an inborn rhythm implying either rhythmic changes in pituitary corticotropin release or diurnal differences in the responsiveness of adrenal cortical cells to it due to changes in mitotic activity of adrenal cortical cells (Perkoff et al, 1959). Adrenal responsiveness to ACTH has been claimed to be less at night than during the day (Perkoff et al, 1959). Cope (1961) suggested that this cycle could reflect either relatively increased adrenal activity or relatively diminished rate of destruction of the adrenocortical hormones in the early part of the day and it is of interest to know that the normal circadian variation in plasma conjugated 17-hydroxycorticosteroids is exaggerated in hyperthyroidism and absent in myxoedema (Martin et al, 1963) and that of free plasma and urinary 17-hydroxycorticosteroids is exaggerated in myxoedema and almost absent in hyperthyroidism, indicating that the thyroid, through its effect on metabolism, and particularly the rate of metabolic transformation of free to conjugated 17-hydroxycorticosteroids, could be partly responsible for this diurnal rhythm. Absence of diurnal variation by measuring plasma 17-hydroxycorticosteroids/

17-hydroxycorticosteroids (Laidlow et al, 1955; Eik-Nes et al, 1955; Lindsay et al, 1956; Doe et al, 1960 a; Ekman et al, 1961), urinary 17-hydroxycorticosteroids (Lindsay et al, 1956) or plasma non-protein-bound cortisol (Doe et al, 1963) has been reported in cases of Cushing's syndrome due to adrenal hyperplasia or adenoma. But cases of simple obesity were reported to retain the normal rhythm (Simkin and Arce, 1962; Schteingart et al, 1963). The absence of diurnal variation in Cushing's syndrome is obviously not due to inability of the adrenal cortices to respond further to increased stimulation by ACTH, as many cases of Cushing's syndrome are known to exhibit a hyper-reactive response to ACTH (Christy et al, 1955; Lindsay et al, 1956; Hinman et al, 1957). According to this hypothesis, non-neoplastic Cushing's syndrome could be caused by a disturbance in the function of an ACTH - controlling hypothalamic centre. This is consistent with the known difficulty of inhibiting endogenous ACTH secretion with glucocorticoids in some patients with Cushing's syndrome due to bilateral adrenal hyperplasia (Liddle, 1960).

Fluctuation from day to day in the adrenal activity of normal persons, as measured by corticosteroid plasma levels (Bliss et al, 1953; Ekman et al, 1961) or urinary excretion (Zondek et al, 1957) also occurs, and can be considerable. This type of variation does not follow a definite pattern and is more striking in cases of Cushing's syndrome (Birke et al, 1956; Bassöe et al, 1958; Doe et al, 1960 a) but was also observed in obese subjects (Baird, 1963).

Variations of adrenocortical activity as regards age also occurs. In the newborn infant, relatively high levels of plasma 17-hydroxycorticosteroids were reported (Ulstrom et al, 1961). Similar observations were reported in urine.

The/

The cause of this rise could either be the increased production of cortisol by the infants adrenals, the mobilisation of cortisol from extravascular sites to the circulation or its delayed metabolism. The fact that urinary excretion of 17-hydroxycorticosteroids is lower on the first day of life than the subsequent days is in favour of the redistribution theory. Half life of cortisol in newborn infants, however, is markedly prolonged (Bongiovanni et al, 1958; Reynolds et al, 1962) as compared to that of the adult (Peterson et al, 1955; Brown et al, 1959; Christy et al, 1959) which indicate decreased rate of cortisol metabolism. This could be due to any of the following possibilities. First, the preponderance of unconjugated polar steroids in neonatal urine, such as 6β-hydroxycortisol, previously mentioned, may reflect the effect of high maternal estrogen on cortisol metabolism, particularly its inhibition of reduction of the A-ring, or that the hepatic enzyme systems of the newborn are immature (Reynolds et al, 1962). A second possibility may be due to the fact that large amounts of progesterone metabolites are excreted in the first day of life and these, including pregnanetriol, inhibit glucosiduronate conjugation (Hsia et al, 1960). ACTH can also produce more secretion of cortisol by the adrenal cortex, inhibit conjugation of steroid metabolites, increase the half life of cortisol (DeMoor et al, 1961 a) and relatively increase 6β-hydroxycortisol excretion (Touchstone and Blakemore, 1961). These observations may indicate that newborn adrenals are under at least moderate ACTH stimulation. The alternate metabolic pathways of cortisol in the newborn have been previously mentioned.

After this early neonatal period, plasma and urine 17-hydroxycorticosteroid levels drop rapidly, only to increase again gradually with age, reaching a maximum/

maximum between 20 and 25 years (Levell et al, 1957; Borth et al, 1957; Appleby and Norymberski, 1957; Clayton et al, 1963). Very slight and gradual decrease of level may follow up to the age of 60, after which this drop may be more pronounced (Baird, 1963). Romanoff and his colleagues (1961), however, using C^{14}-labelled cortisol to estimate cortisol production rate from urine studies in eight young (21-35 years) and eight elderly subjects (65-73 years) showed that the secretion of cortisol and the total excretion of its metabolites THF, allo-THF, THE and cortolone were significantly higher in the younger group. But when they expressed their results in terms of creatinine excretion per day the differences disappeared. Moreover, they showed that in both of these two groups there were essentially similar proportions of the two 11-hydroxyl metabolites (THF and allo-THF) to the two 11-ketone substituted metabolites, THE and cortolone, and of the three 5β- (THF, THE and cortolone) to the one 5α-(allo-THF) cortisol metabolites. They concluded that cortisol metabolism in the two groups is the same and that the quantity of cortisol secreted is related to the muscle mass of the subject. West and his associates (1961) tested the adrenocortical activity in a larger group of subjects with wider age range; namely fifteen young men (25-40 years) and fifty old men (60-96 years). They estimated their plasma levels of free and conjugated 17-hydroxycorticosteroids and urinary excretion of 17-hydroxycorticosteroids. They also assessed the renal, hepatic and thyroid functions of these subjects. They found that there was no significant change in adrenocortical function, as judged by the plasma free and conjugated 17-hydroxycorticosteroid levels, with advancing age, but there was/

was a progressive and significant decrease in the urinary excretion of 17-hydroxycorticosteroids with ageing. Half life of cortisol was longer in the old men and there was a significant increase in the level of blood urea nitrogen, bromsulphalein retention and a significant decrease in endogenous creatinine clearance and basal metabolic rate. The protein-bound-iodine and radioactive iodine uptake by the thyroid were unaffected by ageing. These workers concluded that a combination of decreased cortisol catabolism by the liver and other tissues and an impairment in the renal excretion of cortisol metabolites could explain their findings and that the thyroid gland does not appear to be involved.

Variation of adrenocortical activity in pregnancy also occurs. Elevation of plasma 17-hydroxycorticosteroids (Gemzell, 1953; Bayliss et al, 1955; Birke et al, 1958) and particularly plasma cortisol (Gemzell, 1954; Cohen et al, 1958; Martin and Mills, 1958; Jailer et al, 1959) and an increased excretion of free cortisol in urine (Migeon et al, 1957; Jailer et al, 1959) were reported in late pregnancy. These changes are associated with only slight or moderately increased excretion of urinary cortisol metabolites (Birke et al, 1958; Cohen et al, 1958; Martin and Mills, 1958). The discrepancy between the blood and urinary findings could be explained by retardation of cortisol metabolism in pregnancy (Appleby and Norymberski, 1957; Migeon et al, 1957; Martin and Mills, 1958). These changes are also associated with increased binding capacity of the corticosteroid-binding-globulin to plasma cortisol (Daughaday, 1959; Slaunwhite and Sandberg, 1959; Sandberg and Slaunwhite, 1959; Mills et al, 1960). Since the unbound plasma cortisol is the only active fraction at the tissue level, and since this fraction remains practically constant in pregnancy, the/

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the markedly elevated plasma cortisol levels will not give rise to clinical manifestations of Cushing's syndrome. Doe and his co-workers (1960 b) showed that no increase in al-globulin was noticed but that total and protein-bound plasma cortisol were markedly elevated in pregnant women in the third trimester and that the non-protein-bound fraction of plasma cortisol was significantly but only mildly elevated over normal. They correlated the latter finding with the mild symptoms of adrenal hyperfunction commonly observed in pregnant women, such as the occurrence of purplish striae, glycosuria, hypertension, increased fragility of cutaneous blood vessels, etc. They concluded that in pregnancy there is at least an element of increased adrenocortical activity, thus supporting previous similar reports (Christy et al, 1959; Cope and Black, 1959 b). The increase in the plasma protein-binding of cortisol could explain previously reported delayed cortisol metabolism in pregnancy (Christy et al, 1959) and accordingly the discrepancy between plasma and urinary corticosteroid estimations. This discrepancy may also be explained by the increase of estrogen titre in pregnancy which in turn can lead to inhibition of reduction of the A-ring. Delayed metabolism and prolongation of the biological half life of cortisol with estrogen treatment was frequently reported (Robertson et al, 1959; Peterson et al, 1960; Wallace and Carter, 1960). Administration of estrogen was shown to increase 6β-hydroxycortisol excretion (Lipman et al, 1961). Thus, high titres of estrogen in late pregnancy could explain the previously mentioned increased formation and excretion of 6β-hydroxycortisol and other unconjugated polar metabolites in pregnancy (Frantz et al, 1960).

There are few reports in the literature regarding the <u>adrenocortical activity during/</u>

during the various phases of the menstrual cycle. Staub and his colleagues (1962), however, showed that pregnanediol, the urinary metabolite of progesterone remains at relatively lower levels during and after menstruation till ovulation, when the excretion starts to rise gradually to reach a maximum during the premenstrual period and to drop again just before menstruation. But as progsterone is a common precursor to estrogens and corticosteroids, it can not be clear whether these changes reflect ovarian or adrenocortical activity. Studies of cortisol production rate and metabolism during the various phases of the menstrual cycle are needed to throw more light on this subject.

Inter-relationship of the adrenal cortex and other endocrine glands

The inter-relationship between the adrenal cortex and the pituitary gland has been fully reviewed (see regulation of cortisol secretion).

Hyperfunction of the adrenal cortex was sometimes noticed in the condition called "obese hyperglycaemic syndrome" which is due to hyperplasia of the islets of Langerhans of the pancreas, and adrenocortical dysfunction was also associated with primary dysfunction of the thyroid, ovary and pancreas with apparent normal anterior pituitary function; a rare condition called polyglandular syndrome, or pluriglandular failure. Less than twenty cases have been reported in the literature (Christy et al, 1962) the cause of which is still obscure.

Although the old hypothesis implying that the regulation of secretion, and probably the action, of the adrenal cortex hormones is under the control of the adrenal medulla and its hormones, epinephrine and nor-epinephrine, was recently revived by DeMoor et al (1961 b, 1961 c) it seems to be no longer accepted by most workers nowadays (Forsham, 1962). On the contrary, a permissive role of the adrenal cortex hormones, by which a small amount of adrenocortical hormones should be present, seems to be essential for normal function of the medullary hormones (Ingle, 1954). Nevertheless, on rare occasions, adrenocortical hyperplasia or carcinoma were associated with phaeochromocytoma of the adrenal medulla (Williams et al, 1960; Walters et al, 1962).

Pathologic conditions of the parathyroid glands were also sometimes associated with abnormal adrenocortical function. Hyperfunction of either the parathyroid/

parathyroid or the adrenal cortex can cause bone disease (osteitis in hyperparathyroidism and osteoporosis in hyperadrenocorticism), nephrosclerosis, renal stones but hypercalcaemia in hyperparathyroidism and hypocalcaemia in hyperadrenocorticism. Raker et al (1962) reported the occurrence of primary hyperparathyroidism in association with Cushing's syndrome. They reviewed the few cases in literature connecting primary hyperparathyroidism (parathyroid hyperplasia, adenoma, or even hypercalcaemia or hypercalcaemia and hypophosphataemia) with adrenocortical hyperfunction (hyperplasia or adenoma). In the two patients they examined, they were able to show that hypersecretion of the adrenal cortex did not influence the associated classic blood findings of primary hyperparathyroidism, and that the converse was also true, i.e. that hyperfunction of the parathyroid glands had no influence upon the effect of excess adrenocortical hormones. They concluded that hypercalcaemia in a patient with Cushing's syndrome should be evaluated as in an otherwise normal patient and should not be attributed to the adrenal hyperfunction.

However, the adrenal cortex has been more frequently associated with <u>the thyroid gland.</u> Gabrilove and Weiner (1962) reviewed the cases reported in the literature in whom thyrotoxicosis occurred in patients with Addison's disease and noticed in their patient that as the thyrotoxicosis was controlled, the patient required less cortical substitution therapy to keep the electrolytes within the normal range thus supporting the view of Hellman et al (1961) who, using radioisotopic techniques, noticed an absolute elevation in the endogenous production of cortisol with elevated levels of thyroid hormone and diminished production of cortisol in hypothyroidism, suggesting that the requirement for adrenocortical steroids is increased as the metabolic rate rises. They also noticed/

noticed that in hyperthyroidism cortisol metabolism is shifted towards the 11-ketone metabolites and in hypothyroidism towards the 11-hydroxyl metabolites, suggesting that in thyrotoxicosis the conversion of cortisol to THE represents an increased metabolic inactivation of cortisol. Gold and Crigler (1963) also found an increase in the relative and absolute amounts of THE excreted under the influence of tri-iodothyronine. They also noticed increased rate of steroid-glucuronide formation, increased excretion of total cortisol metabolites, suggesting increased general metabolism of cortisol, but constant 5α- to 5β-metabolites of cortisol. The injection in dog of the active analogues of thyroxin led to accelerated enzymic reduction of the circulating cortisol and increased release of corticotropin (Melby et al, 1960). Hellman and his associates (1961) suggested that patients with thyrotoxicosis might have increased secretion of ACTH, and Hilton and his colleagues (1962) used plasma from patients with thyrotoxicosis to perfuse intact adrenal glands of hypophysectomised dogs. They noticed increased secretion of cortisol in the adrenal venous effluent indicating ACTH-like activity of such plasma. This increase was not noticed when the adrenals were perfused with normal plasma, L-thyroxine, tri-iodothyronine or thyrotropic hormone indicating that the stimulatory substance present in the hyperthyroid plasma could probably be ACTH itself, in which case the action of the thyroid hormone would be to accelerate enzymic reduction and metabolism of the circulating cortisol, thus providing the stimulus to the release of corticotrophin through the negative feed-back mechanism. These workers also found that plasma cortisol levels were slightly less in hyperthyroid than in euthyroid subjects. They concluded that this is not surprising since the stimulus for increased ACTH production comes from the rapid degradation of cortisol, due to the hypermetabolic state, with/

with the result that the increased ACTH produced merely serves to maintain cortisol concentration in the plasma at normal levels. It has been mentioned before that hyperthyroidism accelerates the disappearance of plasma free 17-hydroxycorticosteroids leading to exaggeration of the circadian rhythm of the conjugated 17-hydroxycorticosteroids and that the opposite is true for cases of myxoedema, suggesting that the thyroid gland, through its metabolic effects on free 17-hydroxycorticosteroids may be partly responsible for the circadian rhythm previously discussed.

Chapter II: Methods of assessing adrenocortical function

Estimations in urine

A. Biological methods

The earliest attempts to assess adrenocortical function depended upon extraction of adrenocortical hormones and assessing their activity by administering the extract to, and observing its effects on various laboratory animals.

The activity of the extract was assessed by observing the survival time of adrenalectomised rats on exposure to cold, the degree of deposition of liver glycogen in fasting adrenalectomised rats, the extent of fall of circulating eosinophils in the adrenalectomised mice but the more popular of these early methods measured the degree of sodium retention and potassium excretion in the adrenalectomised rat (Dorfman, 1950).

These methods only confirmed the presence or absence of adrenocortical activity in urine extracts, and could not possibly assess the adrenocortical activity quantitatively. Today they are only of historical interest and they have given way to the more rapid, simpler and more precise chemical methods.

B. Chemical methods

I. Group steroid estimations (see table 1 and 2)

a. Neutral 17-ketosteroids (17-KS)

The earliest chemical methods used to assess adrenocortical function in urine estimated the neutral 17-ketosteroids, sometimes called 17-oxosteroid (or 17-OS), the basic chemical structure of which is either 5 α-androstane or 5β-androstane (etiocholanolone). The main 17-KS compounds in urine are DHA, etiocholanolone and androsterone. Smaller amounts of their 11 oxo- and 11β-hydroxy derivatives are also present, and trace amounts of Δ^4-androstenedione, 11β-/

Table (1)

Methods of estimation of group steroids in urine

Reactions \ Groups	1	2	3	4	5	6	7	8
	21 CH_3 20 C=O 17 C...OH	CH_3 HCOH C...OH	CH_2OH C=O C...OH	CH_2OH HCOH C...OH	O ‖ C	OH | C	CH_2OH C=O C	CH_2OH HCOH C
1 Zimmermann reaction	−	−	−	−	+	−	−	−
2 Reduction by a. Alkaline copper sulphate b. Phosphomolybdic acid or c. Tetrazolium salts	−	−	+	−	−	−	+	−
3 Oxidation by periodic acid (HIO_4)			OH C=O C...OH +HCHO	O ‖ HC C...OH +HCHO			OH C=O C +HCHO	O ‖ HC C +HCHO
Formaldehydogenic steroids	−	−	+	+	−	−	+	+
4 Porter-Silber reaction	−	−	+	−	−	−	−	−
5 BH_4^-	CH_3 HCOH C...OH	CH_3 HCOH C...OH	CH_2OH HCOH C...OH	CH_2OH HCOH C...OH	OH | C	OH | C	CH_2OH HCOH C	CH_2OH HCOH C
6 Na BiO_3	CH_3 C=O C...OH	O ‖ C	O ‖ C	O ‖ C	O ‖ C	OH | C	CH_2OH C=O C	CH_2OH HCOH C
Zimmermann reaction	−	+	+	+	+	−	−	−
7 1. BH_4^- 2. $NaBiO_3$	O ‖ C	O ‖ C	O ‖ C	O ‖ C	OH | C	OH | C	COH | C	COH | C
Zimmermann reaction	+	+	+	+	−	−	−	−
8 IO_4^-	CH_3 C=O C...OH	O ‖ C	COOH C...OH	O ‖ C	O ‖ C	OH | C	CH_2OH C=O C	CH_2OH HCOH C
Zimmermann reaction	−	+	−	+	+	−	−	−

Table (1) contd.

Groups / Reactions	1	2	3	4	5	6	7	8
9 1. BH_4^- 2. IO_4^-	O ‖ C	O ‖ C	O ‖ C	O ‖ C	OH │ C	OH │ C	COH │ C	COH │ C
Zimmermann reaction	+	+	+	+	—	—	—	—
10 1. $NaBiO_3$ 2. BH_4^- 3. $NaBiO_3$	O ‖ C	OH │ C	OH │ C	OH │ C	OH │ C	OH │ C	COH │ C	COH │ C
Zimmermann reaction	+	—	—	—	—	—	—	—
11 1. Zn 2. $NaBiO_3$	CH_3 │ C=O │ C...OH	O ‖ C	CH_3 │ C=O │ C	O ‖ C	O ‖ C	OH │ C	CH_2OH │ C=O │ C	CH_2OH │ HCOH │ C
Zimmermann reaction	—	+	—	+	+	—	—	—
12 CrO_3	O ‖ C	O ‖ C	O ‖ C	O ‖ C	O ‖ C	O ‖ C		
Zimmermann reaction	+	+	+	+	+	+	—	—
13 1. BH_4^- 2. Acid 3. IO_4^-			O ‖ C	O ‖ C	OH │ C	OH │ C		
Zimmermann reaction	—	—	+	+	—	—	—	—
14 1. BH_4^- 2. $NaBiO_3$ 3. HNO_3 4. Fe	—	—	—	—	—	—	(ferric hydroxamates) +	+

11β-hydroxy-androstenedione and adrenosterone are included (see figure 1). Urinary 17-KS are excreted in urine mostly conjugated to sulphuric or glucosiduronic acid (Zumoff and Bradlow, 1963); DHA is almost entirely excreted conjugated with sulphates while etiocholanolone and androsterone as glucosiduronates (Bush and Gale, 1960) and there is reason to believe that 3β-hydroxysteroids in general are conjugated with sulphates and 3 α-hydroxysteroids with glucosiduronates (Staib et al, 1960). 17-KS are either of adrenal or gonadal origin, but 11-oxygenated 17-KS are definitely of adrenal origin. The latter sub-group is either derived from C-19 androgens or indirectly by the oxidative scission of the side chain of the C-21 corticosteroids and their metabolites. In males about two-thirds of the neutral 17-KS is of adrenal origin and one-third of testicular origin. In females almost all the neutral 17-KS are of adrenal origin with the possible contribution of a trace amount from the ovary (Lieberman et al, 1962). The difference in the total neutral 17-KS excretion values between males and females is probably due to the testicular contribution in males (Dorfman, 1960).

The first method for estimation of urinary neutral 17-KS was described by Callow and co-workers (1938). Estimation of total neutral 17-KS involves either preliminary extraction of conjugates or their hydrolysis followed by extraction into an organic solvent, removal of acidic (phenolic) estrogens by alkaline wash and application of a colour reaction.

Hydrolysis of conjugates can be achieved by one of many methods. The first to be used was boiling the urine in acid, hydrochloric or sulphuric, for thirty minutes. Hot acid hydrolysis partly or totally destroys the steroid molecule (Edwards, 1961; Norymberski, 1961) especially in case of glucuronate conjugates, and is not quantitative (Lieberman and Teich, 1953) and in the case of/

of sulphate will also result in marked changes of the steroid molecule which will affect the colour reaction on which the final assay depends (Teich et al, 1953).

Milder methods for hydrolysis of sulphate conjugates were introduced. The first is the continuous ether extraction method of Lieberman and Dobriner (1948) in which urine acidity is adjusted to pH 1 and extraction carried on for three days, steroids freed from sulphates together with unhydrolysed glucuronides can be recovered from the ether layer. The second method is that of Cohen and Oneson (1953) in which 17-KS are extracted in ether-ethanol or butanol, extract evaporated, dehydrated over P_2O_5, then dissolved in trichloracetic acid in anhydrous dioxan and left to stand overnight. Sulphates are hydrolysed and excess acid is removed by alkali wash. The third method is often referred to as the "solvolysis" method of Burstein and Lieberman (1958) in which urine is brought to 2 N with sulphuric acid and steroids extracted three times by ethyl acetate; steroid sulphates hydrolyse if allowed to stand overnight at 37°C.

Few (1961) described another method of hydrolysis using 2.5N sodium hydroxide; hydrolysis was allowed to take place at 37°C for 15 minutes. No destruction of the steroid molecule occurred with this method and hydrolysis was more complete. The enzyme β-glucuronidase is ideal for hydrolysis of the glucuronate conjugates; acidity of the urine is usually adjusted to the optimal pH of the enzyme used and the enzyme added, usually 500 Fishman units to each ml. of urine, and allowed to act from few to 48 hours, usually 24 hours, according to the preparation used. Maximal hydrolysis of 17-KS conjugates is achieved by β-glucuronidase hydrolysis of the glucuronates followed by a hydrolysis of the sulphates by one of the mild procedures described. Some commercial β-glucuronidase preparations were found to yield only 65% or less of the glucuronates of 17-KS (Cohen, 1951; Glenn and Nelson,/

Nelson, 1953; Wotiz et al, 1957). Preparations of β-glucuronidase obtained from the molluscan viscera are highly active (Dodgson and Spencer, 1953). Some preparations of this enzyme, however, namely those obtained from the visceral hump of the common limpet, Patella vulgata, provide β-glucuronidase as well as a sulphatase specific for 3β-sulphates of 5α and Δ^5 steroids (Roy, 1956; Stitch and Halkerston, 1956; Stitch et al, 1956). Moreover, β-glucuronidase obtained from the Roman snail, Helix pomatia, contains β-glucuronidase activity as well as sulphatases for both 3 β-sulphates of 5α- and Δ^5-steroids and 3α-sulphates of 5β-steroids (Leon et al, 1960). No sulphatase activity specific for 3α-sulphates of 5α-steroids (androsterone) was discovered.

Hydrolysis is followed by extraction of the 17-KS into an organic solvent. Ethyl acetate and benzene are popular solvents but it has been noticed that the latter does not extract all 11-oxygenated 17-KS (Edwards, 1961) and that the former extracts contain more contaminating substances.

The crude extract is then washed with alkali to remove phenolic steroids. Drekter and coworkers (1952) were the first to use sodium hydroxide for purification of the extract.

Colour reaction is then applied to the alcoholic solution of the residue and compared to a standard 17-KS, usually DHA. Although several colour reactions have been used, the most commonly used is the Zimmermann reaction (Zimmermann, 1935) in which methylene groups adjacent to ketone groups react with m-dinitrobenzene in strong alkali. Sodium hydroxide was first used, but it was noticed that colour reaction with alcoholic potassium hydroxide is more stable than alcoholic sodium hydroxide (Wilson and Carter, 1947). Tetramethylammonium hydroxide renders this colour reaction even more stable and much less sensitive to light (James and deJong, 1961). In 17-KS the active methylene group/

group is at C-16 and reacts with meta-dinitrobenzene producing a violet colour with maximum extinction at 520 mμ. (reaction 1, table 1). This reaction could be applied to steroids on paper chromatograms; non-polar steroids give a blue colour, relatively polar ones a dirty grey colour, and 5α-steroids a violet colour. Although urinary 17-KS constitute the great majority of all urinary substances giving this reaction (Bush, 1961), a few other chromogenic substances with ketone groups remain in the extract after the alkali wash thus contributing to the end result. Further modification of the shade and intensity of the colour produced can also occur in the presence of ketone groups other than at C-17 in the 17-KS molecules themselves, i.e. at C-3 and C-11, as well as in the presence of 11β-hydroxy group. Multiple carbonyl groups have a summation effect and hydroxyl groups inhibiting effect on the colour reaction (Edwards, 1961; Bush, 1961). For this reason elimination of the effect of the remaining interferring material is often achieved by measuring the colour intensity at the appropriate wavelength and at two other wavelengths equidistant from it and applying an appropriate correction formula (Talbot et al, 1942; Engstrom and Mason, 1943; Allen, 1950).

Other types of colour reactions have been used, e.g. the antimony trichloride reaction (Pincus, 1943 b) in which all four saturated 3-hydroxy-17-KS give a blue colour with a maximum extinction at 610 mμ. Only faint colour is given by DHA, while pregnanediol and other non-ketonic steroids as well as some 17-hydroxy C-19 steroids contribute to the colour produced (Edwards, 1961). A third colour reaction is the Pettenkofer reaction originally described by Pettenkofer (1844). A blue colour, with maximum extinction at 660 mμ., is produced with 3 β-hydroxy- Δ^5-steroids in the presence of furfural, acetic acid and/

and sulphuric acid at 67°C. Munson and co-workers (1948) applied this method to estimate DHA, the principal substance in urine estimated by this method.

Normal values of urinary neutral 17-KS differ in the hands of various workers but a range of 6-25 mg./24 hours for adult males, 20-40 years old, and 3-20 mg./24 hours for females (Loraine, 1958) is accepted by most authors. In patients with adrenocortical hyperfunction 17-KS level is generally increased if androgen production is affected, since only 5-8% of cortisol is metabolised as 17-KS (Sandberg et al, 1957 a; Edwards, 1961). In Addison's disease and in pituitary disorders which affect ACTH release, 17-KS production is diminished.

The use of chromatography could separate 11-deoxy- from 11-oxygenated-17-KS and this may be a more sensitive index of adrenocortical function which could be helpful in differentiating normal values from those of Cushing's syndrome (James, 1961). Goldzieher and Axelrod (1962) using paper chromatographic analysis claimed that up to 90% of the clinical total 17-KS may consist of non-specific chromogens. The specificity of 17-KS estimation is diminished by the inevitable presence of artifacts in the final residue; moreover it measures androgenic more than corticosteroid activity because only a small percentage of cortisol is metabolised as 17-KS. This means that 17-KS excretion should be considered as a measure of the weakly androgenic secretion of the adrenal cortex and not as a measure of overall adrenocortical activity. Even as a measure of androgenicity the 17-KS are only a rough estimate, since the individual compounds differ widely in their androgenic potencies. Thus the usefulness of this estimation as an index of adrenocortical activity is only limited to the finding of a gross departure from the normal.

In this study, however, a 17-KS method based on that described by James and deJong (1961) was used and compared to other methods assessing adrenocortical function./

function.

b. Group corticosteroids (see table 2)

The C-21 corticosteroid estimations should give a better idea of the adrenocortical function. The chemical reactions of corticosteroids depend upon the type of side chain attached to C-17. It must be noticed that, unlike 17-KS, corticosteroids side chain is very labile in strong acid or alkali solutions (Edwards, 1960) and for this reason β-glucuronidase hydrolysis is often resorted to. These chemical reactions may be discussed under the following headings.

1. Reducing corticosteroids

Substances with α-ketol groups which include steroids with this functional group at C-20:C-21 [$-CO.CH_2OH$] are reducing in nature. Talbot and co-workers (1945) applied this fact to estimate total reducing corticosteroids in urine containing this group using alkaline copper sulphate reagent. Heard and Sobel (1946) used phosphomolybdic acid reagent and claimed an enhancement of the reaction in the presence of Δ^4-3-ketone group, and Chen and Tewell (1951) used a tetrazolium salt (2:3:5 - triphenyl tetrazolium chloride) in alkaline medium. Corticosteroids estimated by this method are; cortisol, cortisone, compound S and their dihydro and tetrahydro-derivatives, DOC, compound B, compound A and their dihydro- and tetrahydro-derivatives and aldosterone. (see reaction 2, table 1).

The alkaline copper sulphate and phosphomolybdic acid methods mainly estimate unconjugated reducing corticosteroids and moreover these reduction reactions in general can include estimations of other non-steroidal reducing substances in the urine which could not be removed even with preliminary purification./

Table (2)

Urinary estimations of group corticosteroids

Author	Method	Values mg./24 hours	
		Males	Females
Patterson (1961)	Lowenstein et al (1946) Enzyme hydrolysis, formaldehyde.	3 - 26	
Glenn and Nelson (1953)	Enzyme hydrolysis, Porter-Silber reaction.	3.3 - 9.3	2.1 - 5.0
Reddy (1954)	Porter-Silber reaction without hydrolysis	2.9 - 12.0	1.1 - 8.6
Norymberski et al (1953)	Bismuthate oxidation, acid hydrolysis	9.6 - 19.2	4.6 - 13.4
James and Caie (1964)	Few (1961) Borohydride reduction, periodate oxidation, alkaline hydrolysis	7.6 - 19.5	4.9 - 15.1

purification. Thus due to the lack of specificity of these procedures serious errors in calculations may result. Moreover, important metabolites of cortisol, e.g. cortols and cortolones, are not included in this estimation. Because of all these disadvantages, these techniques are no longer popular. The tetrazolium salts, however, especially 3,3′ - dianisole bis-4,4′ (3,5-diphenyl) tetrazolium chloride (= blue tetrazolium), are very widely and successfully used now, only after isolation of individual steroids by chromatography, for detection and estimation of corticosteroids. They give bright red or blue coloured formazans with α-ketol groups.

2. Formaldehydogenic steroids

These are corticosteroids with a hydroxy -methyl group at C-21- adjacent to either a ketone or another alcoholic group [$-CO.CH_2OH$ or $-CHOH.CH_2OH$] with or without a hydroxyl group at C-17. They can react with periodic acid to yield one molecule of formaldehyde per molecule of corticosteroid. Corticosteroids estimated by this method are; cortisol, cortisone, compound S and their dihydro- and tetrahydro-derivatives, α- and β-cortol, α- and β-cortolone, DOC, compound B, compound A and their dihydro- and tetrahydro- and 20-dihydro-derivatives and aldosterone (see reaction 3, table 1). The original technique based on the reaction of chromotropic acid with formaldehyde (MacFadyen et al, 1945) was described by Lowenstein and co-workers (1946) and was later improved and modified by Daughaday et al (1948), Corcoran and Page (1948) and Hollander et al (1951). Formaldehyde is liberated from the reaction mixture by steam distillation, trapped in sulphite solution and allowed to react with chromotropic acid, giving a violet colour. Disadvantages of this technique are many. First, the reaction is not specific; any contaminating/

contaminating compound with hydroxymethyl group adjacent to a ketone or another alcohol group which could not be removed by preliminary washing can give this reaction (Patterson, 1961). Secondly, some urinary contaminants can bind formaldehyde inhibiting either its liberation or its reaction with chromotropic acid. Thirdly, the technique is difficult to standardise (Patterson, 1961) and adjustment of the acidity at the beginning of the test to pH 1 leads to marked losses of the total metabolites of corticosteroids. An advantage of this method, however, is that it estimates practically all metabolites of cortisol and corticosterone and none of their precursors. Figures given by this method, 3-26 mg./24 hours for normal subjects, are often higher than those given by other methods, and on the whole, on account of the great disadvantages of this method it is abandoned nowadays.

3. Acetaldehydogenic steroids

Similar to the previous method, periodate oxidation of C-21 steroids with 21-methyl-17,20 dihydroxy side-chain [-COH.CHOH.CH_3] results in liberation of acetaldehyde which can also be quantitatively estimated by a colour reaction. This method first introduced by Cox (1952) measures only pregnanetriol (and its 11-keto derivative) and Δ^5-pregnenetriol and may be of value in diagnosis of adrenogenital syndrome where deficiency of 21- or 11-hydroxylase enzymes in the adrenal cortex results in blocking of cortisol synthesis and increased excretion of the metabolites of its precursors.

4. Porter-Silber chromogens (P-S chromogens)

These are corticosteroids with dihydroxy-acetone side chain [-COH.CO.CH_2OH] often called 17-hydroxycorticosteroids especially in the U.S.A. To avoid confusion between this method and that described by Norymberski (see later) a better terminology/

terminology is 'Porter-Silber chromogens' or 'dihydroxyacetonyl steroids'.

This group of corticosteroids in alcoholic solution react with phenylhydrazine in the presence of sulphuric acid to give yellow coloured osazones with maximum extinction at 410 mμ. The corticosteroids estimated by this method, therefore, are; cortisol, cortisone, compound S, their dihydro- and tetrahydro-derivatives (see reaction 4, table 1). This reaction was first described by Porter and Silber (1950) but was applied in two different ways. Reddy and co-workers (1952) extracted the free and conjugated corticosteroids directly into butanol, the extract was subjected to a short step purification and the Porter-Silber reaction carried out directly on the corticosteroids contained therein. This method is rapid and simple but butanol contains impurities which give positive Porter-Silber reaction and even when redistilled butanol is used this method is still thought of as a crude method not to be used for estimating normal levels or in research work but only suitable where exceptionally high results of corticosteroids are expected (Patterson, 1961). Glenn and Nelson (1953) however, performed this method by applying hydrolysis of conjugates using β-glucuronidase as the first step followed by extraction of the corticosteroids into chloroform, purification by alkali wash and florisil columns. The organic solvent for extraction could be ethyl acetate, methylene chloride or ethylene dichloride and more purification could be achieved by chromatographic separation of the corticosteroids. Patterson (1961) summarised the disadvantages of this method in two main points. First, only the main group of cortisol metabolites are estimated, while cortols and cortolones for example are not included. Secondly, many drugs interfere with the test e.g. iodine,/

iodine, potassium iodide, quinine, sugars, ascorbic acid, paraldehyde, chloral hydrate, sulphamerazine and senna. Nevertheless Patterson stated that with careful attention to the details, this method, as far as adrenocortical function is concerned, can provide the basis of a sound and accurate method for group analysis better than any routine 17-KS method in accuracy and reproducibility.

Normal figures given by this method are; for the Reddy technique, 2.9-12.0 mg./24 hours for males and 1.1-8.6 mg./24 hours for females; and for Glenn and Nelson technique, 3.3-9.3 mg./24 hours for males and 2.1-5.0 mg./24 hours for females. As it is known that about one third of the cortisol secreted by the adrenal cortex is estimated as urinary Porter-Silber chromogens (Forsham, 1962), multiplying the figure obtained from urinary measurement by three yields the approximate twenty-four hours adrenal secretory rate.

5. 17-ketogenic steroids (17-KGS)

It has been shown that three groups of corticosteroids can be oxidised by sodium bismuthate to yield 17-KS (Norymberski, 1952; Norymberski et al, 1953). These groups were termed 17-ketogenic steroids, and are sometimes referred to as 17-oxogenic steroids (see reaction 6, table 1). These three groups are; the dihydroxyacetone group [$-COH.CO.CH_2OH$] which includes cortisol, cortisone, compound S and their dihydro- and tetrahydro-derivatives; the 17,20,21-trihydroxy- group [$-COH.CHOH.CH_2OH$] which includes the cortols, cortolones and pregnanetetrol; and the 17,20-dihydroxy-21-methyl group [$-COH.CHOH.CH_3$] which includes pregnanetriol (and its 20β-hydroxy-isomer, 5α-isomer, 5α-20β-hydroxy-isomer, 5α-3β-hydroxy-isomer, and 5α-20β-hydroxy-3β-hydroxy-isomer), 11-keto-pregnanetriol and Δ^5-pregnenetriol. The original method described implies oxidation of the urinary corticosteroids by bismuthate carried out in 50% acetic acid, to 17-KS; hydrolysis of conjugates by hot acid hydrolysis, extraction and application of Zimmermann reaction. If 17-KS were/

were estimated on another aliquot of the same urine, the difference between the two estimations will give the value for the 17-KS formed by bismuthate oxidation, i.e. the 17-KGS.

In 1955, the Norymberski group (Appleby et al) extended their research a step further. They showed that sodium borohydride has marked reducing properties on the carbonyl groups at C-11, C-17, and C-20. Thus it can transform the dihydroxyacetone group [$-COH.CO.CH_2OH$] to the 17,20,21-trihydroxy group [$-COH.CHOH.CH_2OH$] and the 17-hydroxy, 20-keto, 21-methyl group [$-COH.CO.CH_3$] to the 17,20-dihydroxy-21-methyl group [$COH.CHOH.CH_3$] as well as the 17-KS [-CO] to 17-hydroxysteroids [-COH] (see reaction 5, table 1). They applied this finding to their previous technique. Thus by applying sodium borohydride reduction followed by sodium bismuthate (see reaction 7, table 1), the naturally occurring 17-KS in urine will not be included in the final result, while the 17-KS formed will be derived from all 4 groups of corticosteroids containing a hydroxyl group at C-17 and accordingly this method estimates total 17-hydroxycorticoids, better called <u>total 17-ketogenic steroids</u> (total 17-KGS). Thus this method will estimate the groups and compounds mentioned in the previous technique plus an extra group, the 17-hydroxy, 20-keto, 21-methyl group [$-COH.CO.CH_3$] which includes 17α-hydroxypregnanolone (and its 5α-isomer and its 5α-3β-hydroxy-isomer), 17α-hydroxyprogesterone and the di- and tetrahydro-derivatives of both 21-deoxycortisol and 21-deoxycortisone (see figure 1). A great advantage of this modification is that the initial reduction eliminates the pre-existing 17-KS and thus one final colorimetric determination is needed. This increases markedly the precision of the technique. Another advantage is that/

that borohydride reduction also eliminates other non-specific ketonic chromogens which could give a positive colour reaction. Potassium borohydride is cheaper, less hygroscopic and as efficacious as the sodium salt.

Appleby and Norymberski (1955) used borohydride and bismuthate in the sequence: oxidation-reduction-oxidation (see reaction 10, table 1). The resulting 17-KS formed are derived only from the 21-deoxyketols, i.e. 17-hydroxy, 20-keto, 21-methyl group [$-COH.CO.CH_3$].

The same group of workers (Exley et al, 1961) devised a similar technique to estimate corticosterone and its metabolites in urine. Thus they subjected the urine to the sequence: reduction-oxidation (see reaction 14, table 1) which results in transforming the groups; $-C.CO.CH_2OH$ and $-C.CHOH.CH_2OH$ into C-20 aldehydes, which could be converted into the corresponding hydroxamic acids, which in turn could be transformed into their purple ferric hydroxamates. This technique estimates the compounds; DOC, compound B, compound A, their dihydro- and tetrahydro- and 20-dihydro-derivatives and aldosterone.

Norymberski and Stubbs (1956) showed that the dihydroxyacetone group can be specifically attacked by zinc. Subsequent oxidation by bismuthate (see reaction 11, table 1) leads to estimation of the 17,20-dihydroxy-21-methyl and the 17,20,21-trihydroxy groups together with the pre-existing 17-KS. The difference between this estimation and that of the original bismuthate method gives an estimate of the important dihydroxyacetone group.

Norymberski (1961) also showed that oxidation with chromic anhydride alone converts all 17-hydroxycorticosteroids and 17-hydroxyandrostanes to 17-KS (see reaction 12, table 1) thus the total 17-KGS, 17-KS and 17-hydroxyandrostanes could be estimated at the same time by the Zimmermann reaction.

Although the original 17-KGS method is superior to the Porter-Silber technique/

technique at least as far as its being able to estimate more cortisol metabolites besides its being more simple, yet many drawbacks have been noticed. The method as a whole suffers from two inevitable disadvantages; the first is that it measures pregnanetriol and so this technique will be of limited value in diseases such as the ~~androgenital~~ adrenogenital syndrome (Kent et al, 1963). Secondly, a considerable lack of precision appears with subnormal corticosteroid levels in urine. The use of bismuthate further adds to the disadvantages of the method. Few (1961) summarised these disadvantages. They include the deleterious effect on DHA and interference with 17-KS estimation, both of which, however, could be avoided by the use of borohydride. Serious underestimations occur in the presence of excess sugars in the urine. This could also be avoided by using larger amounts of bismuthate. Duration of use of bismuthate is also important since some batches could transform 11β-hydroxy-17-KS to 11-keto-derivatives. It is known that 11β-hydroxy-etiocholanolone and its 5α-isomer, 11β-hydroxy-androsterone, are the two 17-KS which should be formed in this reaction. But it was observed that in the sequence borohydride reduction-bismuthate oxidation-acid hydrolysis 11-oxoetiocholanolone and Δ^{9-11}-etiocholanolone were formed by oxidation and then dehydration, by hot acid hydrolysis, of the 11β-hydroxy-etiocholanolone initially formed. This could be avoided by using β-glucuronidase or periodic acid for hydrolysis. It was also shown that 11β-hydroxy-etiocholanolone can be formed from corticosteroids not possessing oxygen atom at C-11, and that this can be included in the final result thus severely limiting the accuracy of the quantitative evaluation. Another disadvantage of bismuthate oxidation is that only about 80% could be recovered from the starting material. A last disadvantage in the use of bismuthate is the variation of reactivity with the type/

type of bismuthate used.

Tompsett and Smith (1955) were the first to use periodate oxidation for elimination of all the disadvantages of bismuthate, but they included a hot-acid hydrolysis step between the reduction and oxidation steps and they conducted the periodate oxidation in a strongly acid medium. Few (1961) believes that these acid conditions are likely to cause the partial dehydration of 11β-hydroxysteroids. He, therefore, used sodium meta-periodate and alkaline hydrolysis. Periodate alone transforms only the 17,20,21-trihydroxy and 17,20-dihydroxy, 21-methyl groups to 17-KS (see reaction 8, table 1) but if the sequence: sodium borohydride - sodium meta-periodate is applied all four 17-hydroxycorticosteroids are transformed to 17-KS (see reaction 9, table 1). Thus Few estimated the total 17-KGS by applying sodium borohydride then sodium meta-periodate followed by mild alkaline hydrolysis and extraction into ether. The extract was purified by chromatography, partition column using Celite, to separate 11-deoxy- from 11β-hydroxy 17-KS. Using this method all the disadvantages of using bismuthate were eliminated and if any formates of 11β-hydroxy-etiocholanolone were formed they did not survive the alkaline hydrolysis and were transformed back to 11β-hydroxy-etiocholanolone. But still pregn-5-ene-triol could be detected which might have formed some artifacts, and the 11β-etiocholanolone could be formed from 11-oxopregnanetriol thus emphasising the fact that this method also will be of limited use in diseases like the adrenogenital syndrome.

Rutherford and Nelson (1963), applying the sequence sodium borohydride reduction - sodium meta-periodate oxidation proposed by Few (1961) with chloroform as the extracting solvent, noticed marked improvement in reproducibility over the bismuthate/

bismuthate method. James and Caie (1964) reported that the method of Few compared to estimations of cortisol production rate by C^{14}-labelled cortisol studies is satisfactory as regards precision and accuracy, but the correlation became poor as the secretion rate increased.

Michelakis (1962) resorted to a preliminary step of separating urinary 17-KS from urinary C-21 corticosteroids by using benzene-water partition; 17-KS and contaminating material were extracted in the benzene layer and C-21 steroids were separated into the water layer. This was followed by the sequence sodium borohydride reduction - periodate oxidation and the two 11β-hydroxy-17-KS formed were extracted into either benzene, diethyl ether or ethylene dichloride and the estimations performed using the Zimmermann reaction. High recoveries and better reproducibility and specificity were also reported by this method; only less than 5% of the cortisol metabolites, excreted as 11-oxygenated 17-KS, will not be measured.

In most instances 17-KGS and total 17-KGS methods will give identical results since the contribution of the 21-deoxyketols in the normal subject is negligible (Norymberski, 1961). But either of them should give higher results than the Porter-Silber method; the difference should mainly represent the contribution of cortols and cortolones. There are many advantages of those indirect methods of corticosteroid estimation, as a whole, over the direct Porter-Silber one. First, the reaction sequence to which the steroids are subjected ensures a considerable degree of specificity especially when it involves both reduction and oxidation steps. Secondly, the labile acid and alkali sensitive side-chain is degraded into a stable 17-carbonyl group which simplifies subsequent manipulations. Thirdly all the known major metabolites of cortisol are/

are estimated by these methods. Lastly all these metabolites are converted into a mixture of 11β-hydroxy-etiocholanolone and 11β-hydroxy-androsterone of which the latter is only a minor component, or may even be lacking altogether in some cases. These two compounds have the same chromogenicity in the Zimmermann reaction, which enables accurate estimates of the excretion of cortisol metabolites to be made.

Normal levels of 17-KGS, which could also represent total 17-KGS (Norymberski, 1961), are 9.6-19.2 mg./24 hours for males and 4.6-13.4 mg./24 hours for females (Norymberski et al, 1953). Figures given by the method of Few (1961) are nearly similar; namely 7.6-19.5 mg./24 hours and 4.9-15.1 mg./24 hours for males and females respectively. Multiplying the estimated figures by two gives an approximate value for twenty-four hour secretory rate of cortisol (Cope, 1961; Forsham, 1962).

In this study total 17-KGS were estimated by a method based on that described by Few (1961) and compared to other indices of adrenocortical function.

6. Conjugated corticosteroids

Very recently Zumoff and Bradlow (1963) isolated urinary conjugates of corticosteroids as a group by a method based on the ability of albumin to bind steroid conjugates. Thus albumin was added to urine and co-precipitated with ammonium sulphate. Precipitated albumin was separated by centrifugation and conjugates of injected C^{14}-labelled cortisol were eluted from the protein by denaturation with organic solvents. The authors of this method claimed better recovery of corticosteroid conjugates than the usual acid or enzyme hydrolysis procedures. The method is simple, not expensive, time saving and may well be suited for routine use.

II/

II. Individual steroid estimations

a. Cortisol excretion

The estimation of the unmetabolised primary hormone, cortisol, as excreted unchanged in urine is of a great biological significance. This is usually achieved by extraction of the acidified urine, with chloroform, ethylene dichloride or methylene dichloride, and the application of chromatography. The use of C^{14}-labelled cortisol, to allow estimation of losses during the procedure, renders the quantitative estimation (usually carried out by the Porter-Silber reaction or fluorometrically) very accurate.

Only a very small proportion of the endogenous cortisol is excreted unchanged in urine; and the values given by various workers range between 0-250 µg./24 hours (Burton et al, 1951 b; Cope and Hurlock, 1954; Bongiovanni, 1958; Ross, 1960; Kornel and Hill, 1961; Dohan et al, 1962; Rosner et al, 1963). This estimation is the urinary reflection of the biologically active unbound plasma cortisol. Mills and co-workers (1959) found that circulating cortisol was 95% bound when the plasma concentration was 10 µg./100 ml., 80% at plasma concentration of 40 µg./100 ml. and only 72% at a plasma concentration of 80 µg./100 ml. Assuming a glomerular filtration rate of for example 100 ml. /minute, at plasma concentration of 10 µg./100 ml. and 95% protein binding only 0.5 µg. of cortisol is filtered per minute, while at plasma concentration of 80 µg./100 ml. and 72% protein-binding 22.4 µg. of cortisol is filtered per minute. Thus there is a forty-five fold increase in the filtered load for an eight-fold increase in plasma concentration.

Even though 80-90% is reabsorbed from the renal tubules in normal subjects (Schedl/

(Schedl et al, 1959) there is sufficient urinary loss to afford a sensitive indicator of free physiologically active plasma cortisol. Cope and Black (1959 a) also suggested that detection of increased excretion of unmetabolised cortisol in the urine may be a more sensitive index of raised blood levels than the measurement of the total urinary corticosteroids. Similarly in cases of Cushing's syndrome, there is an increase in total plasma cortisol level, disproportionate increase in the unbound fraction, increased excretion of metabolites and a disproportionate and very significant increase of the excretion of unmetabolised cortisol (Greaves and West, 1960; Kornel and Hill, 1961). This is presumably because of the weaker binding of large quantities of the circulating cortisol to albumin and the subsequent rise in glomerular filtration rate. Contrary to the belief that unmetabolised cortisol is excreted unconjugated (Peterson et al, 1955; Neher, 1959) recent studies proved that it could be conjugated as glucosiduronate or sulphate at C-21 (Pasqualini, 1960; Pasqualini and Jayle, 1961).

Very recently, Rosner and co-workers (1963) estimated unmetabolised cortisol excretion using glass fibre chromatography and corrected for losses by C^{14}-labelled cortisol. Besides confirming the clear separation between normal values and those found in adrenocortical hyperactivity states, they also observed that no difference existed between figures of males and females, no diurnal variation existed in the pattern of unmetabolised cortisol excretion and marked daily variations existed in cases of Cushing's syndrome. They enumerated the advantages of this test. As a diagnostic tool, it clearly separates normal values from those found in adrenocortical hyperfunction and also differentiates cases/

cases of Cushing's syndrome from obesity, the latter with normal excretion of unmetabolised cortisol. Secondly, it is more sensitive than other methods of measurement of adrenocortical function including estimations of the biologically active fraction of plasma cortisol. Thirdly, conditions like thyrotoxicosis may lead to increased hepatic enzyme activity leading, in turn, to accelerated degradation of cortisol with compensatory increase in cortisol production to values well within the range found in Cushing's syndrome. The normal excretion of unmetabolised cortisol in such conditions excludes the diagnosis of Cushing's syndrome. Lastly it is superior to plasma cortisol estimations where emotional upset may cause transient high values which would not be reflected in 24 hour urinary collection.

b. Excretion of individual metabolites of cortisol

This line of study is of extreme importance in the knowledge of the various metabolic pathways of cortisol. It is also important in determinations of specific activity of the various metabolites necessary for estimating cortisol production rate by the radioisotope studies (see later). Hydrolysis and extraction of corticosteroids are carried out as previously described. It should be noted here that polarity of steroids increases by increase in number of ketone groups and increases more with increased numbers of hydroxyl groups, and that solvents also show a gradation in polarity from the unsubstituted saturated hydrocarbons such as light petroleum through the aromatic hydrocarbons, benzene, ethylene dichloride, ethers, ketones, ethyl acetate, chloroform and alcohols to water (Brooks, 1961). Steroids are most soluble in solvents of similar degree of polarity. Apart from using this information to extract steroids, it can be used also to separate them/

them by applying two immiscible solvents to the steroid mixture one of which should be most suitable for extracting a particular steroid, i.e. with a high partition coefficient for that steroid, a method used in "counter-current separation".

Separation, however, is best achieved by partition chromatography, either on column or better still and easier on paper together with a standard steroid on a separate lane. Two systems of paper chromatography are often used; the Bush system (Bush, 1952) is based on a volatile solvent as the stationary phase, namely methanol, and impregnation of the filter-paper occurs entirely via the vapour phase. This is in contrast to the methods developed by Zaffaroni and his co-workers (Burton et al, 1951 a) where paper is first impregnated with a non-volatile solvent to provide the stationary phase of the chromatogram. The disadvantage of the first system is its great sensitivity to temperature changes and that of the second system is the difficulty in removing the stationary phase at the end of the run. Detection of the steroid with the Δ^4-3-ketone grouping, characteristic of cortisol and other active adrenal steroid hormones, on the chromatograms can be achieved by visualising them as dark spots on exposure to ultraviolet light of wavelength 254 mμ. Other steroids can be detected on paper by the Zimmermann reaction or better still by blue tetrazolium. Quantitative estimation of the steroid can be done by elution of the part of the chromatogram containing it in a suitable solvent, the eluted steroid then being estimated photometrically in solution using the absorption or fluorescence of the compound itself or that of a reaction product. Alternatively the steroid can be measured by treating the chromatogram with reagents which form coloured/

coloured or fluorescent spots with the compounds on paper; the intensity of the spots is measured photometrically (Bush, 1960).

Estimations in blood

A. Biological methods

These, as in the case of urine studies, were the oldest methods for estimating adrenocortical activity. They included prolongation of the life of adrenalectomised dogs by injection of serum (Anderson and Haymaker, 1938) and the cold protection test in rats (Vogt, 1943), etc. Today these tests also have given way to the more accurate and simpler chemical tests.

B. Chemical tests

I. Methods for the determination of corticosteroids or cortisol in blood

In blood, unlike in urine, cortisol is the main corticosteroid, constituting over 80% of the circulating corticosteroids (Mason and Sprague, 1948; Bush and Sandberg, 1953; Romanoff et al, 1953; Hudson and Lumbardo, 1955). Bush and Sandberg (1953) applied chromatographic methods to separate cortisol in peripheral blood and identified this compound by several criteria including light absorption, reaction with tetrazolium salt, fluoresence in sodium hydroxide and degradation to the expected 17-KS.

Methods for estimation of adrenocortical activity in blood are very limited; they could be either crude methods of estimating corticosteroid levels or more refined for the estimation of cortisol levels after chromatographic separation. As the concentrations of steroid in peripheral blood are very low, it is particularly important from the analytical point of view, especially in cortisol estimations, that any degree of contamination or interference should be minimised.

a. Collection of blood samples

Standardised times are important since a diurnal rhythm of cortisol level in blood is well established (Lewis, 1957). Blood from fasting patients is preferred/

preferred to avoid interference by lipid material in the plasma, especially with some of the refined techniques (Braunsberg and James, 1960). An anticoagulant is usually used, most often heparin. Oxalates may be used, but citrates interfere with colorimetry in the method of Nelson and Samuel (1952).

Plasma, not serum, should be used since redistribution of steroids occurs between the red cells and plasma during the clotting process and low levels have been reported in serum (Bongiovanni, 1954). Eik-Nes and co-workers (1953) stated that migration of steroids into red cells takes place if blood is allowed to stand, an observation confirmed by Peterson and his associates (1955) using C^{14}-labelled cortisol. DeMoor and Meulepas (1962) also showed that increased removal rate of corticosteroids from plasma was under certain circumstances due to an increased storage of plasma corticosteroids within or on the red blood cells. Whole blood also is unsuitable for estimation of corticosteroids, since it is very difficult to extract cortisol from lysed cells (Bush, 1957). Once separated from the blood cells, the plasma can be stored at 4°C for a short time (Nelson et al, 1951) or deep frozen for longer periods (Harwood and Mason, 1956), but it must be noticed that some denaturation of protein occurs on deep freezing (Brown et al, 1957).

b. Extraction of corticosteroids

There is no evidence to indicate that precipitation of proteins offers any advantage over direct extraction. Pre-wash of plasma to remove lipid with carbon tetrachloride (Silber and Busch, 1956), iso-octane (Silber et al, 1958) or petroleum ether (Moncloa et al, 1959) prior to extraction, produces considerably cleaner/

cleaner extracts. The solvent of choice for extraction is methylene chloride, because of its greater stability (Bongiovanni and Eberlein, 1955; Silber and Busch, 1956). One extraction with 5 volumes of methylene chloride yields about 98% of the cortisol in plasma (Peterson et al, 1957 a). Ethyl acetate and chloroform (Nelson and Samuels, 1952) may be used; two extractions with 1.5 volumes of chloroform appear to be adequate (Harwood and Mason, 1956).

Metabolic products of cortisol, like the tetrahydro-derivatives, occur mostly in the conjugated form in blood. They are thus water soluble and are not extracted by these lipid solvents.

Dialysis procedures, which are supposed to provide an extract which is relatively free from lipid and pigment, do not offer any great advantage over straight-forward extraction methods; further purification is still desirable.

c. Purification of corticosteroids

The use of solvent partition, especially benzene/water partition, achieves considerable purification of cortisol (Eik-Nes, 1957). To remove some steroidal estrogens and other phenolic and acidic compounds, an alkali wash of the organic solvent extract is incorporated in some methods. One-fifth to one-tenth volume of 0.1N sodium hydroxide is commonly used (Silber and Porter, 1954; Silber and Busch, 1956). Potassium carbonate and sodium carbonate were used (Bush and Sandberg, 1953) but none of these methods effectively removes the less polar estrogens such as estrone and estradiol.

Crude chromatographic purifications on "Florisil", a commercial magnesium silicate preparation (Nelson and Samuels, 1952), magnesium silicate/Celite (Nelson and Samuels, 1952), reverse phase partition (Morris and Williams, 1953 b) and silica gel columns (Bush and Sandberg, 1953) have been used in some methods. It/

It is not known whether or not these offer advantages over simple solvent partitions. On the other hand they need careful preparation and variable losses may occur. However, among these crude chromatographic purification methods, that using silica gel offers advantages of simplicity and reproducibility.

d. Separation of Cortisol

The more refined methods resort to separation of cortisol by chromatography. Generally speaking, fractionation of the steroids is brought about by adsorption and/or partition chromatography. In column adsorption chromatography, a mixture of steroids in solution is passed through a finely divided adsorbent; the various steroids are adsorbed at different distances along the length of the column, and may be separated, either by cutting the column and dissolving each steroid in a suitable solvent, or by passing a solvent through so that the various steroids pass out of the bottom of the column in an order which is dependent upon their physical properties and the adsorption of the column. In column partition chromatography, the column is impregnated with one solvent (the stationary phase) and the mixture of steroids, dissolved in a second solvent (the mobile phase), is passed through it. The steroids are separated in an order depending not upon the adsorption but upon their relative solubilities in the two solvents. This principle is also applied in paper partition chromatography, where filter paper is used as the support for the stationary phase.

Column adsorption chromatography, e.g. silica gel adsorption columns (Sweat 1954 b) has been used for determination of steroids in blood (Sweat, 1955), but it did not give reproducible results; "tailing" occurred and the steroid fractions from plasma extracts were contaminated with other compounds which interfered with subsequent determination (Takeda, 1956).

Column/

Column partition chromatography was first used by Morris and Williams (1953 b) to separate adrenocortical steroids. They used columns containing a stationary phase of 25% aqueous ethanol with toluene as eluent. Excellent recovery of cortisol was achieved by these methods, but exceptionally high values of corticosterone plasma levels were obtained. Many useful systems have, however, been derived from this method, and their application to blood extracts has met with some success (Ayres et al, 1957; Braunsberg and James, 1960).

Paper partition chromatography, on the other hand, has been used extensively for separation of cortisol and other steroids in blood. Crude plasma extracts have been fractionated on either the Bush (Bush, 1952) or the Zaffaroni (Burton et al, 1951 a) systems. The latter are capable of handling larger quantities and thus are more suitable for relatively impure extracts, and offer advantages for larger blood volumes.

The use of paper chromatography presents two difficulties. First, the value of the "blank" is often high and has to be reduced by adequate washing of the paper before use, to ensure that the blank is sufficiently low and uniform over the length of the paper; secondly, the problems of determination of steroids _in situ_ after chromatography or quantitative elution from paper prior to assay, which in most cases still leads to a lower degree of precision than does, for example, the use of partition columns. However, trace amounts of isotopically labelled steroids, added to blood before extraction, help to obviate this difficulty (Avivi et al, 1954; Peterson, 1957; Bondy and Upton, 1957). Paper chromatography on the other hand offers an advantage when large numbers of samples are to be analysed.

Counter/

Counter-current distribution which has been applied to urinary extracts and extracts from adrenal vein blood was not applied to peripheral blood. Counter-current separation of a large number of samples, however, is inconvenient and time consuming.

e. Determination of corticosteroids (see table 3 and 4)

1. Oxidation methods

As in the case of urine, periodic acid can affect oxidative release of one formaldehyde molecule per molecule of steroid containing a hydroxymethyl group at C-21 [$-CH_2OH$]. Corcoran and Page in 1948 published a method based on this principle for determining corticosteroid levels in plasma and urine. They extracted heparinised plasma with acetone after precipitation of plasma proteins by an alcohol-ether mixture, periodic acid was added and the formaldehyde liberated estimated after reaction with chromotropic acid. This method, although very simple, is nonspecific; phospholipids, traces of which could not be eliminated produced formaldehyde, and other steroids containing the C-21 hydroxymethyl group also produced formaldehyde. Thus, values given by this method were very high; 110-420 µg./100 ml. and this method is no longer used.

2. Ultraviolet absorption methods

Light absorption at 240 mµ. is typical of Δ^4-3-ketosteroids. However, reagent background, other steroidal and non-steroidal compounds may give absorption in this region, necessitating a considerable degree of purification. Also, the molecular extinctions are lower than those produced in some colour reactions, necessitating the use of larger blood volumes (Weichselbaum and Margraf, 1955).

3. Colour reactions

i. Porter-Silber reaction. The alcoholic solutions of steroids containing the dihydroxy-acetone group, with some degree of specificity, react with phenyl-hydrazine/

Table (3)

Methods for estimation of corticosteroids in plasma

Author	Sample	Extraction	Purification	Determination	No. of Subjects	Age (years)	Sex	Time a.m.	Value (μg./100 ml.)	
									Mean and S.D.	Range
Corcoran and Page (1948)		alcohol:ether (protein precipitation), acetone	Petroleum ether	Periodic acid oxidation. Estimation of liberated formaldehyde by chromotropic acid						110.0-420.0
Bliss et al (1953) [Method of Nelson and Samuels (1952)]	30ml. blood or 10 ml. plasma	4 x 1 vol. 4:1 ether: chloroform or 3 x 1.5 vol. chloroform	Hexane/70% ethanol or benzene/water partition and magnesium silicate/ Celite or Florisil columns	Porter-Silber reaction in 0.5 ml. final vol. Allen correction: 370, 410, 450 mμ.	(91 (29	20-45 20-45	M F	8:00-8:30	12.0± 6.0 15.0± 6.0	2.0-34.0 2.0-31.0
Silber and Porter (1954)	10ml. plasma	1 x 2.5 - 5 vol. chloroform	1 x 2 ml. 0.1 N NaOH wash	Porter-Silber reaction directly on 1 ml. final vol. - 410 mμ - (plasma H_2SO_4 as blank)	16				13.3±6.2	6.0-25.0
Bierich (1959) [Method of Silber and Busch (1956)]	10ml. plasma	Pre-wash with carbon tetrachloride, 2.5 vol. and petroleum ether, 2.5 vol. Extraction with methylene chloride 1 x 2.5 vol., 1 x 1 vol.	NaOH wash	Porter-Silber reaction directly on 1 ml. final vol.	25	3-15		9:00-11:00	10.3±4.5	2.4-23.5
Peterson et al (1957 a)	0.2ml. plasma	1 x 5 vol. chloroform	NaOH wash	Porter-Silber reaction directly on 1 ml. final vol.	50				15.0±4.5	6.0-25.0
DeMoor et al (1960 b) [Method of Silber et al (1958)]	0.2 - 2 ml. plasma	Pre-wash with petroleum ether, 3 vol., extract with methylene chloride, 3 vol.	0.2 vol. 0.1 N NaOH	Extraction of solvent with 30 N H_2SO_4 and fluorescence read after 30-90 minutes	(23 (20	25-39 25-39	M F	8:00-9:00 8:00-9:00	18.4 23.0	

Table (4)

Methods for estimation of cortisol in plasma

Author	Sample	Extraction	Purification	Separation	Determination	No. of subjects	Age (years)	Sex	Time a.m.	Value (µg./100ml)	
										Mean & S.D.	Range
Morris and Williams (1953 b)	20ml. plasma	4 vol. ethanol	Partition in 20% ethanol/ethylacetate and 50% ethanol/ carbon tetrachloride	Celite partition chromatography	Polarography of hydrazones	(4 ((3 (	20-45	M F		8.4±1.5	6.5-10.5
Sweat (1955)	5ml. plasma	chloroform	70% ethanol/petrol-eum ether partition	Silica gel chromatography	Fluorescence in sulphuric acid	21		M		10.8±2.6	
Braunsberg and James (1960)	10ml. plasma	(pH of plasma adjusted to 9.0± 0.5)2 x 3 vol. ethyl acetate	Silica gel column	Celite partition chromatography	Fluorescence in ethanol sulphuric acid	(7 ((6	18-51	M F	9:30-10:30	7.8±2.4	4.8-13.3
Lewis (1957)	5ml. plasma	2 x 4 vol. ethyl acetate	alkali wash	paper chromatography	Fluorescence in sulphuric acid	(20 ((10	15-42	M F	8:00-9:00	9.2±1.5	6-12
Bondy and Upton (1957) Bondy et al (1957)	(20ml. plasma (+C^{14}-cortisol (+C^{14}-cortico- (sterone	add alkali. 3 vol. chloroform		paper chromatography	Radioactivity and fluorescence in pot. t-butoxide	(29 ((33				10.2±3.6 8.1±3.6	4.0-17.7
Bojesen (1956)	5-10ml. plasma	1 x 1.5 - 3 vol. chloroform	70% ethanol/ hexane partition	paper chroma-tography after esterification with 'pipsan' -S^{35}	Radioactivity	6			10:00	13.3	6.9-20.0
Berliner (1957)	5-10ml. plasma	Chloroform	75% methanol/ hexane partition or heptane pre-wash	paper chroma-tography after C^{14}-acetylation	Radioactivity	6			3:00p.m.	8.6±0.4	
Murphy et al (1963)	1ml. plasma	-	-	equilibrium dialysis for C^{14}-unbound cortisol	Radioactivity compared to standard protein-binding curve						7.4-15.4
Osman (1961)	1-2ml. plasma	(pre-wash with petroleum ether) methylene chloride	alkali wash	-	Fluorescence in ethanol sulphuric acid of extract and standard cortisol and corticosterone	(15 ((12		M F	8:00-9:00	9.6±2.7	5-15

hydrazine in sulphuric acid yielding yellow coloured osazone derivatives with peak absorption at 410 mμ (Porter and Silber, 1950; Silber and Porter, 1954). In normal persons, cortisol accounts for approximately 90% of the unconjugated plasma corticosteroids that give this reaction (Peterson et al, 1955) and the unconjugated dihydro-derivatives accounts for most of the rest of them. Steroids other than cortisol with 20-keto-17,21-dihydroxy grouping may contribute to this colour reaction and so do α-β-unsaturated ketones like compound S and even non-steroidal compounds such as sugars, ascorbic acid, aldehydes, ketones, quinine, colchicine, potassium iodide, chloral, paraldehyde, chlorpromazine, sulphamerazine, bilirubin, etc. (Braunsberg and James 1961). Thus cortisol can sometimes account for as little as two-thirds or even one third of the Porter-Silber colour produced by plasma extracts (Bayliss and Steinbeck, 1953). Accordingly Allen's correction formula was frequently used to eliminate or minimise this source of error (Allen, 1950). A related reaction with 2,4-dinitrophenylhydrazine is more sensitive but less specific (Gornall and MacDonald, 1953); most steroids produce considerable colour, and absorption peaks are not sufficiently different to allow determination of individual compounds.

The use of these methods applied to crude preparations from blood is, therefore, not likely to yield meaningful results, but they may be useful after separation and careful purification. Methods using the Porter-Silber reaction include Nelson-Samuels method (Nelson and Samuels, 1952), which needs considerable training and care but has low specificity for cortisol and is not very sensitive; the lowest measurable blood concentration is 5 μg./100 ml. It can reflect grossly increased or decreased adrenocortical activity but is not suitable for precise/

precise and accurate determination of cortisol.

Silber and Porter (1954) suggested a method not involving chromatographic purification, and using direct extraction of corticosteroids into the Porter-Silber reagent. Its sensitivity and specificity are not better than the Nelson-Samuels method, and its precision remains low at low levels; this is why the originators of this method added 5 μg. cortisol to the extract, to increase the sensitivity of the method, and subtracted this amount later from the final result.

ii. Reduction of "blue tetrazolium" (3:3'-dianisole-4:4'-3:5-diphenyltetrazolium chloride) in alkaline solution, by α-ketols (Mader and Buck, 1952), to purplish or mauve-coloured water-insoluble pigments called formazans was applied to the determination of cortisol and other corticosteroids in peripheral blood (Chen et al, 1955). Sodium hydroxide, choline or tetramethyl-ammonium hydroxide were used to provide an alkaline medium. Although many steroids not containing the α-ketolic grouping react more slowly, these do not usually interfere with determination of adequately purified cortisol. Thus, the sensitivity of this method slightly exceeds that of the Porter-Silber reaction, but its use for crude blood extracts (Weichselbaum and Margraf, 1955) must be condemned since it has low specificity. Elution of the chromatograms either precedes (Vogt, 1954) or follows (Cope et al, 1955) reaction of the steroid with the tetrazolium salt, or the paper may be added to the reagent (Chen et al, 1955). Sensitivity of these methods is reduced by the paper blank, and the method is thus difficult to apply to the small amounts of steroid available from peripheral blood.

iii. Reaction with isonicotinic acid hydrazide of Δ^4-3-ketosteroids produces hydrazones with absorption maxima at 380 mμ. (Umberger, 1955), and was suggested as /

as a basis for determining physiologically active corticosteroids in plasma (Weichselbaum and Margraf, 1957). The sensitivity of this method is considerably lower than that based on direct ultraviolet absorption, but the difference in the rates at which the various ketones condense with this reagent, and in the absorption maxima, lend some specificity to the method. Compounds other than α-β-ketones may, however, contribute to the reading at 380 mμ.

4. Fluorometric methods

Cortisol and corticosterone undergo reactions in alkaline media (Bush, 1952; Abelson and Bondy, 1955) and sulphuric or phosphoric acid (Wintersteiner and Pfiffner, 1936; Sweat, 1954 a; Goldzieher et al, 1954; Goldzieher and Besch, 1958; Kalant, 1958) with the formation of unknown products, which can be measured fluorometrically. Fluorescence in alkali is specific to Δ^4-3-ketones (Bush, 1952) and high specificity of fluorescence in acid requires a Δ^4-3-ketone group and a hydroxyl group at C-11 (Sweat, 1954 a). Interference by impurities also occurs but careful preparation and purification of the samples and the attention to details of fluorescence development, activation and measurement may reduce any interference. The availability of highly sensitive photomultipliers makes it possible to use these methods for very dilute solutions, and the fluorescence is directly proportional to concentration when the extinction is 0.05 or less (Braunsberg and Osborn, 1952). This is the range of concentrations in which absorptiometric methods become inaccurate. The use of fluorometric techniques, therefore, seems attractive for work with small quantities of steroids such as those present in blood.

Fluorescence reactions in alkaline or acid media can be applied to steroids on paper after chromatography (Bush, 1952; Zaffaroni and Burton, 1951) or in solution (Abelson and Bondy, 1955; Sweat, 1954 a; Goldzieher et al, 1954). Fluorometric methods can thus be applied with success to purified fractions from human/

human peripheral blood, but their use for crude plasma extracts raises problems of specificity. The introduction of a simple and rapid technique using fluorometric methods for the estimation of cortisol and corticosterone levels in crude extracts of peripheral blood, by making use of special curves to exclude interfering substances (Osman, 1961), was a great achievement towards increasing the specificity and simplicity of these methods.

Methods involving fluorometric determinations include that of Silber et al (1958). In this method a minimum of purification is adopted and the method depends almost entirely on the specificity of the fluorescence reaction. Although high precision, accuracy and sensitivity were described for this method, 'residual fluorescence' appears to be fairly constant, reviving the problems of specificity, and so these methods may be useful for following changes in plasma levels of cortisol, or only employed after separation of cortisol in silica gel adsorption columns, paper partition chromatograms or Celite partition columns.

For separation of cortisol, adsorption on silica gel has been used (Sweat, 1955). Florisil columns were used prior to silica gel chromatography (Ely et al, 1958), but unknown contaminants lead to lowering of the specificity of this method. This, together with poor reproducibility, render the use of this method very limited. Celite partition chromatography (Braunsberg and James, 1960) of cortisol is lengthy and exacting and liable to interference by impurities. Paper chromatographic methods were used by some workers to separate cortisol. Lewis (1957) employed a preliminary reverse phase chromatogram to remove less polar contaminants from plasma fractions, followed by a benzene/50%-methanol chromatographic system. The steroids were determined by sulphuric acid fluorescence after elution. In other similar methods (Bondy et al, 1957; Bondy/

Bondy and Upton, 1957) steroids were determined by estimating their fluorescence in potassium t-butoxide and radioactive steroids were added to the plasma to allow for losses in the isolation procedures. Specificity of these paper chromatographic methods depends upon the resolving power of the paper system and the fluorescence technique. High precision was described for these methods, and they are reasonably specific for cortisol, but high sensitivity is achieved only when paper blanks are sufficiently low or constant.

5. Polarographic methods

These were first introduced by Morris and Williams (1953 a). Although this technique gave recoveries of 80%, it has not found further application because it requires critical purification of each compound.

6. Isotopic methods

There are three methods utilising radioactivity measurements for determination of corticosteroid levels in blood. The first involves the addition of a known amount of isotopically labelled steroid to the sample, and radioactivity measurement of the final fraction submitted to quantitative chemical analysis. This constitutes an internal standard to correct for all losses occurring during chemical manipulations. Thus, in those methods, manipulations need not be quantitative resulting in saving of time, and the assessment of accuracy is more valid than that based on conventional recovery experiments. Greater precision can be achieved if the quantity of tracer compound added is sufficiently small to be ignored in the final determination. This requires tracers of very high specific activity. This method has been applied to the determination of cortisol in plasma (Bondy et al, 1957).

The second application of tracer isotopes is in quantitative reaction of the steroid with a suitable reactive reagent to yield a chemically stable derivative, the/

the radioactivity of which is a measure of the amount of steroid present. The reaction in this method must be highly specific, or a rigorous purification procedure must be adopted to isolate the required compound. Acetylation with C^{14}-acetic anhydride (Berliner, 1957; Hollander and Vinecour, 1958), or the reaction with S^{35}-labelled-'pipsan' (p-iodophenyl-sulphonylic acid anhydride) (Bojesen, 1956) followed by paper chromatography were applied to the determination of cortisol in plasma.

A combination of the previous two methods utilising two different and distinguishable radioactive isotopes, one for chemical reaction with steroids and the other for correction of losses during the procedure, is called the 'double tracer technique' and can offer high precision, accuracy and sensitivity (Avivi et al, 1954). A third application of the radioisotopes was demonstrated by Murphy and co-workers (1963). These workers made use of the steroid binding capacity of the plasma, by adding increasing amounts of unlabelled cortisol to an equilibrium dialysis system containing a standard plasma and a constant amount of C^{14}-labelled cortisol. This led to decrease in the proportion of the percentage of protein-bound C^{14}-labelled cortisol from which a standard curve could be drawn. The plasma to be tested was similarly treated, and its cortisol content could be determined from the standard curve. The authors of this method reported high degrees of reproducibility and specificity for cortisol; the method was not affected by drugs or haemolysis. Recoveries were 91%, and only 1 ml. plasma was required. Its precision was high at low levels (0-10 μg./100 ml.), and many samples of blood could be estimated at a time which rendered the method especially useful in adrenocortical stimulation and suppression tests.

Examination/

Examination of table 3 and table 4 reveals that the methods employing the Porter-Silber reaction give similar means and ranges, and that techniques employing careful chromatographic separation of cortisol produce mean values which are slightly lower than those obtained by the cruder methods. It must be noted, however, that no appreciable difference between sexes could be demonstrated (Bliss et al, 1953; Lewis, 1957; DeMoor et al, 1960 b), although some data suggest that cortisol values in women may be lower than in men (Bondy and Upton, 1957).

In summary, the inclusion of an adequate chromatographic stage appears to be necessary at present, if reliable estimation of cortisol in blood is to be made. Since small volumes of blood are used, the question of sensitivity is important. Isotopic techniques are capable of the highest possible precision and sensitivity. Also, the sensitivity of the fluorimetric methods is relatively high. The development of methods for the determination of cortisol in plasma has been prompted to a large extent by the desire to obtain a chemical measure of the hormonal activity to which tissues are subjected. However, other factors, e.g. the diurnal rhythm, variation in response to stress and emotional upsets, day-to-day fluctuation of plasma levels, function of the thyroid gland and liver, and the extent of protein-binding of plasma corticosteroids, must modify our concept of the relationship between these chemical methods and the biological activity of corticosteroids. Therefore, the interpretation of plasma steroid levels must be made with caution, since hormonal activity may not be properly reflected by the concentration of circulating steroid.

II./

II. Methods for the determination of plasma-protein binding of cortisol

Soon after the discovery that cortisol and other corticosteroids circulate in the plasma bound to proteins, and with the knowledge that the organic solvents used in the classical methods of estimation of cortisol level in blood extract both the bound and unbound cortisol, several methods were proposed to estimate the degree of binding of cortisol and to correlate it, and apply it as a diagnostic measure, to the various clinical dysfunctions of the adrenal cortex. The oldest of these methods (Eik-Nes et al, 1954 b) was based on the difference in 'solubility' of steroids in saline and albumin solutions. Other methods were based on dialysis of plasma (Bush, 1957; Daughaday, 1958 a; Slaunwhite and Sandberg, 1959; Sandberg and Slaunwhite, 1959); ultraviolet spectrophotometry of steroids containing the Δ^4-3-keto group (Westphal, 1957); electrophoresis of the plasma and separation of the various protein fractions and identification of the steroids attached to them (Westphal et al, 1955; Daughaday, 1958 b; Slaunwhite and Sandberg, 1959; Sandberg and Slaunwhite, 1959) or ultrafiltration of the unbound fraction and estimation of its proportion to the total cortisol in plasma (Sandberg et al, 1957 b; Chen et al, 1958; Mills et al, 1959).

The method based on solubility did not give a true picture of the binding in plasma, since cortisol in particular is mainly bound to globulin, and that based on ultraviolet absorption showed a marked overlap of the steroid peaks in the ultraviolet spectrum. All the previously mentioned methods, however, were only either qualitative for the detection of the unbound fraction of cortisol, or at the best semi-quantitative.

More recently, Mills and his associates (Mills et al, 1960; Chen et al, 1961;/

1961; Mills, 1961 a; Mills, 1962) improved the ultrafiltration technique to measure the degree of cortisol binding in a truly quantitative manner. These workers added C^{14}-labelled cortisol in a small quantity, so chosen that the ratio of bound to unbound cortisol was not disturbed even at the lowest concentration of steroid, i.e. they added a small amount of cortisol of high specific activity. Plasma was then transferred to the visking tubing and ultrafiltration was carried out by centrifugation at 37°C overnight. The proportion of radioactivity of a certain volume of the ultrafiltrate, representing the unbound cortisol, to the same volume of total plasma, representing both the bound and the unbound cortisol, was a measure of the proportion of the unbound to the total cortisol in plasma.

In 1962, Daughaday and his co-workers improved their method of measuring the binding capacity of the corticosteroid-binding globulin (CBG). They devised a double equilibrium dialysis procedure based on differential heat inactivation of the CBG at 4°C and 60°C, thus rendering it more quantitative. The CBG-binding capacity as estimated by this method was 20.1 ± 5.2 µg./100 ml., with no significant difference between males and females. Murphy and Pattee (1963) found it easier and more accurate to measure the CBG-binding capacity in terms of the amount of cortisol required to saturate it. They thus modified the dialysis method and carried out their estimations at 37.5°C. They obtained higher values, but also without significant sex difference (30.9 ± 4.5 and 31.8 ± 5.5 µg./100 ml. for females and males respectively). These workers noticed no diurnal variation of CBG-binding capacity and no effect of ACTH on it.

The ultrafiltration technique, however, has many advantages over the dialysis methods./

methods. It is rapid, simple and is carried out at 37°C which renders the technique more accurate from the physiological point of view. The use of radioisotopic cortisol gave high precision to the method, even at low concentrations of plasma unbound cortisol, but most important is the fact that only this technique measures <u>directly</u> the unbound fraction of cortisol in plasma, while the dialysis methods measure the CBG-binding 'capacity', and, as Daughaday pointed out (Daughaday et al, 1962), no correlation was observed when CBG-binding capacity was plotted as a function of the plasma 17-hydroxycorticosteroids.

Quantitative estimation of the degree of binding by plasma proteins to cortisol, has been used to assess if it could be of any diagnostic help in adrenocortical or other dysfunctions. Under normal conditions, i.e. at physiological level, 90-95% of the cortisol in plasma is bound and 5-10% is unbound (Mills et al, 1960). Doe et al (1960 b) reported a significant rise of the unbound fraction after ACTH stimulation, and in cases of adrenocortical hyperfunction due to bilateral hyperplasia. More recently Doe and his associates (1963), using a dialysis technique and C^{14}-labelled cortisol, showed that overlap occurs between the percentage of non-bound cortisol in normal subjects and in patients suffering from Cushing's syndrome, but pointed out that if the absolute level of the unbound fraction is taken into consideration no overlap will be noticed. These workers showed for the first time a circadian rhythm of the non-protein-bound cortisol in normal subjects (with values of 1.2-2.7 µg./100 ml. at 9:00 a.m. and, 0.12-0.74 µg./100 ml. at 9:00 p.m.) which was absent in cases of Cushing's syndrome. So, it seems that study of the binding of plasma proteins to cortisol is more promising/

promising as far as it is throwing more light on the problems of understanding and diagnosis of adrenocortical dysfunctions. In this thesis, a method based on the ultrafiltration technique was studied as an index of the adrenocortical function.

Estimation of cortisol production rate (see table 5)

It has been mentioned before that plasma cortisol levels should be interpreted with care because of the many factors which influence them. This means that these estimations are not sensitive indicators of the actual adrenocortical activity.

Attempts to estimate the actual production rate of cortisol by the adrenal cortex started in 1952 when Knowlton successfully maintained Addisonian patients on 12.5-25 mg. of cortisone per day and suggested that the actual daily output of the normal gland must be of the same order. Later, Bondy and Altrock (1953) estimated 17-hydroxycorticosteroids simultaneously in the plasma of the renal artery and renal vein and calculated the production rate by multiplying the difference of the two values by the value of the renal plasma flow. They obtained values in normals of 15.0-25.0 mg./24 hrs. Hardy and Turner (1957) estimated cortisol production rate by simultaneous determination of cortisol secreted in the adrenal vein and the adrenal vein blood flow. They reported values of 34.0 mg./24 hrs.

Although the last two approaches were more direct ones, yet these methods were performed under abnormal conditions and therefore are only of very limited use. Dorfman (1954) applied a different approach. Observing the proportion of an ingested dose of cortisol excreted as 11-deoxy- and 11-oxy-metabolites of 17-KS in urine, calculated a production rate of 21.0 and 18.0 mg./24 hrs. for males and females respectively. Silber (1955) administered graded doses of cortisol and calculated the production rate by subtracting the amount of metabolites estimated by the Porter-Silber reaction, excreted/

Table (5)

Estimations of cortisol production rate

Author	Method	Values mg./24 hours	
		Males	Females
Knowlton (1952)	Replacement therapy	12.5 - 25.0	
Bondy and Altrock (1953)	Simultaneous estimation of: 17-hydroxycorticosteroids in renal artery and vein, and renal plasma flow.	15.0 - 25.0	
Hardy and Turner (1957)	Simultaneous estimation of cortisol in adrenal vein and adrenal vein blood flow.	34.0	
Dorfman (1954)	Excretion of 11-oxy- and 11-deoxy- neutral 17-KS after test dose.	21.0	18.0
Silber (1955)	Excretion of 17-hydroxy-corticosteroids after test dose.	21.9 ± 8.3	
Moxham and Nabarro (1956)	Excretion of P-S chromogens and 17-KGS after test dose.	(P-S 14.0-45.0 (17-KGS 8.5-49.0	9.5 - 36.0 5.5 - 40.0
Samuels et al (1957)	Rate of disappearance of administered cortisol.	36.0	
Peterson and Wyngaarden (1956)	Isotopic dilution: blood method.	12.0 - 29.0	
Cope and Black (1958 a)	Isotopic dilution: urine method.	4.9 - 25.3	

excreted at basal (or control) period from that excreted after the test dose and expressing it in terms of cortisol. His value for males were 21.9 ± 8.3 mg./24 hrs. Moxham and Nabarro (1956) used the same approach to correlate an oral cortisol dose with excretion of either 17-KGS or Porter-Silber chromogens, and their values calculated from 17-KGS were 8.5 - 49.0 and 5.5 - 40.0 and from Porter-Silber chromogens 14.0 - 45.0 and 9.5 - 36.0 mg./24 hrs. for males and females respectively.

Samuels and co-workers (1957) were the first to measure the rate of disappearance of infused cortisol, as estimated by the plasma cortisol level, and to apply it to calculate the production rate, which was about 36.0 mg./24 hrs. All the methods described so far, however, are based ultimately on estimation of cortisol in blood, and so, beside the difficulties in performing some of them, they suffer from lack of precision due to incomplete enzymatic hydrolysis, incomplete extraction, losses during the procedure and other analytical difficulties. Measurement of production rate by estimating the disappearance rate of infused cortisol is the nearest approach to a sound technique, but the administration of cortisol in measureable amounts most probably disturbs the normal distribution and metabolism of cortisol, and thus renders the physiological significance of the test dubious.

It was only by the use of isotopically labelled cortisol that accurate estimation of cortisol production rate was made possible. In the blood method (Peterson and Wyngaarden, 1956) the principle of the technique is to administer intravenously a trace amount of C^{14}-labelled cortisol, in a quantity so small that it will not affect the metabolism or plasma level of endogenous cortisol./

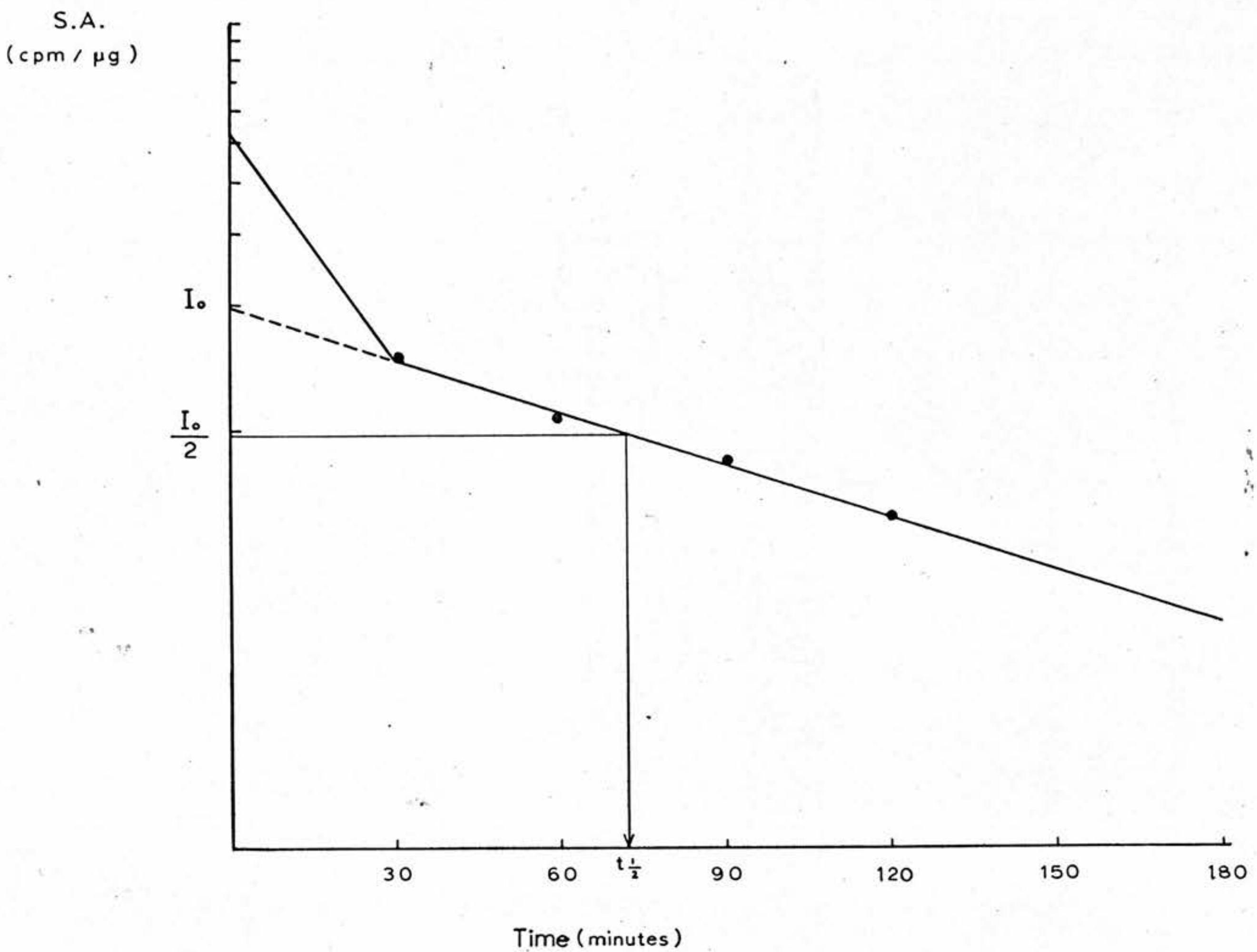


FIGURE 4: Semilog. plot of S.A.

cortisol. Plasma samples are withdrawn at half hourly intervals. Plasma cortisol is extracted and purified by chromatography. Both the radioactivity and quantity of the purified cortisol extract are determined from which the specific activity is calculated (radioactivity divided by quantity). Because the radioactive cortisol is continuously removed from the body, the specific activity of the first sample is highest and that of the fourth sample is lowest. The rate of turnover of cortisol at a unit time is proportional to the amount of cortisol in the body, because turnover of cortisol follows an exponential or logarithmic manner. Therefore, if the specific activities of the four purified cortisol extracts were plotted on a semilogarithmic graph against the time of withdrawal of the corresponding blood samples, they fall more or less on a straight line (see figure 4). If this straight line is extrapolated back to zero time, it will provide the zero time specific activity (Io, expressed as counts per minute/µg., or cpm/µg). Io, therefore, represents the degree of dilution of the radioactivity of the administered C^{14}-cortisol into the endogenously produced cortisol at zero time had the mixing of the radioactive and non-radioactive cortisol been instantaneous.

If Ii is the specific activity of the C^{14}-cortisol dose solutions, also in cpm/µg., then the ratio $\frac{Ii}{Io}$ represents the degree of dilution of the administered amount of cortisol (a, in µg.) in the body cortisol at zero time (Ao, also in µg.).

$$\text{Thus} \quad \frac{Ao}{a} = \frac{Ii}{Io}$$

$$\text{or} \quad Ao = a[\frac{Ii}{Io}]$$

But the amount of cortisol present in the body at zero time, Ao, equals the/

the endogenously produced cortisol present in the body (A, in μg.) plus the trace amount of injected labelled cortisol. Therefore, the endogenously produced cortisol, also called the body miscible pool of cortisol, can be calculated from the isotope dilution expression:

$$A = a\left[\frac{Ii}{Io}\right] - a$$

$$\text{or } A = a\left[\frac{Ii}{Io} - 1\right] \ \mu g.$$

$$\text{or } A = a\left[\frac{Ii}{Io} - 1\right] \times 10^{-3} \quad mg. \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \ (1)$$

To estimate the biological half clearance time, the value $\frac{Io}{2}$ is calculated and a line drawn horizontally from that point on the ordinate of the graph to meet the line of fit at a point, from which another line is drawn vertically to meet the abscissa at a point representing the biological half clearance time ($t\frac{1}{2}$).

To estimate the cortisol production rate two values are needed to be known:

a. the miscible pool of cortisol in the body

b. its rate of turnover

Calculation of the miscible pool has already been discussed.

To estimate the rate of turnover we assume that

Fo is the amount of cortisol present in the body initially,

F the amount of the original cortisol present in the body at a given time,

δF the amount of cortisol replaced by endogenously produced cortisol in a short period (δt minutes)

Therefore:-

The rate of turnover $= \frac{\delta F}{\delta t}$

It has been mentioned that the rate of turnover at a unit time is proportional/

proportional to the amount of cortisol in the body because turnover follows an exponential or logarithmic manner. Therefore:-

$$-\frac{\delta F}{\delta t} = kF \qquad \text{where } k = \text{turnover constant}$$

The negative sign means that a given amount of cortisol secreted in the body diminishes by time.

Upon rearrangement of the last equation we get,

$$\frac{\delta F}{F} = -k\delta t \qquad \text{Therefore,}$$

$$\int \frac{1}{F}\, dF = \int -kdt + M \qquad \text{Where M is the integration constant.}$$

Carrying out the process of integration we get

$$\log_e F = -kt + M \qquad \text{Where e is the base of natural logarithms.}$$

But, when $t = 0$, $F = Fo$ Therefore, the last equation could be rearranged to get:

$$\log_e Fo = M \qquad \text{and} \qquad \log_e F = -kt + \log_e Fo$$

$$\therefore \log_e F - \log_e Fo = -kt$$

$$\therefore \log_e \frac{F}{Fo} = -kt$$

$$\therefore \frac{F}{Fo} = e^{-kt} \qquad \text{or} \qquad F = Fo\, e^{-kt}$$

Conversion of this equation for the first order reaction to ordinary (Briggsian) logarithms is made by introducing the conversion factor 0.4343 so that last equation could be written:

$$\log \frac{F}{Fo} = -0.4343\ kt$$

When/

When we want to estimate the half clearance time,

$\frac{F}{Fo} = \frac{1}{2}$ and $t = t\frac{1}{2}$ so last equation could be written:

$$\log \tfrac{1}{2} = -0.4343 \ kt\tfrac{1}{2} \quad \text{or} \quad \log 2 = 0.4343 \ kt\tfrac{1}{2}$$

$$\text{or} \quad 0.3010 = 0.4343 \ kt\tfrac{1}{2} \quad \text{or} \quad \frac{0.3010}{0.4343} = kt\tfrac{1}{2}$$

Therefore, $k = \frac{0.693}{t\frac{1}{2}}$ pool/minute

This last equation expresses the rate at which the body miscible pool of cortisol is replaced by newly synthesised endogenous cortisol. It can be written:

$$k = \frac{0.693}{t\frac{1}{2}} \times 60 \times 24$$

$$= \frac{997.9}{t\frac{1}{2}} \text{ or approximately } \frac{1000}{t\frac{1}{2}} \text{ pool/24 hr} \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

The product of the miscible pool, A, and the rate of turnover, k, i.e. (1) x (2), gives the production rate in mg./24 hours.

In proposing this method Peterson and Wyngaarden proved the validity of the various assumptions that had to be made before accepting this technique. These assumptions were:-

1. That the injected cortisol is pure.
2. That the injected cortisol does not significantly alter the size of the miscible pool and does not disturb the normal metabolism of endogenously produced cortisol.
3. That the size of the miscible pool remains constant during the period of the study and that the rate of synthesis of cortisol equals the rate of transformation.
4. That the administered radioactive cortisol is handled like the endogenous steroid/

steroid and that there is a random disappearance of both the endogenous and labelled cortisol.

5. That the mixing of the injected steroid is homogeneous within the pool and is rapid compared with its metabolism.

6. That the rate of metabolism of cortisol is proportional to its concentration.

7. That the radioactive steroid does not re-enter the pool.

8. That the isolated plasma cortisol is pure.

Nowadays, practically the only assumption that has to be checked by workers using this method is that the miscible pool remains constant during the period of the test by comparing cortisol plasma levels of the first and last samples withdrawn. This of course assumes that a reliable batch of cortisol of great purity is used, and that a reliable method of purification of the extracted plasma cortisol is used. The main advantage which this technique offered, besides giving the correct information about the actual state of activity of the adrenal cortex, is its high degree of precision; the volume of plasma samples, completeness of extraction and recovery of cortisol did not matter because only the specific activity i.e. the proportion of radioactive to non-radioactive cortisol is measured, and as this proportion is constant throughout the test only the very last step of the technique i.e. the estimation of the specific activity, needs to be strictly quantitative. Cortisol production rate of normal subjects by this method was estimated to be 12.0-29.0 mg./24 hrs.

Cope and Black (1958 a) applied the same principle to estimate cortisol production rate from urine studies. The radioactive cortisol dose could either be given intravenously or orally and a twenty-four hour urine sample collected./

collected. The total radioactivity of urine was measured and metabolites of cortisol in urine were hydrolysed and the specific activity of the major metabolite THE estimated. If C_u is the total radioactivity in urine and $S.A._F$ the specific activity of the administered dose then the ratio $\frac{C_u}{S.A._F}$ equals the amount of radioactive cortisol present in the urine in the form of all metabolites. If $S.A._u$ is the specific activity of the urine metabolites, then $\frac{S.A._F}{S.A._u}$ equals the degree of dilution of the administered cortisol with the endogenously produced cortisol, and the production rate will be

$$\frac{C_u}{S.A._F} \times \frac{S.A._F}{S.A._u} = \frac{C_u}{S.A._u}$$

The specific activity of THE, the main metabolite of cortisol, is representative of the specific activity of all cortisol metabolites and so cortisol production rate = $\frac{C_u}{S.A._{THE}}$ mg./24 hrs.

The validity of the same assumptions of the blood method are also applied to the urine method. Other factors, however, may contribute to a low excretion of the radioactive cortisol in the urine method and thus affect the measurement of the true production rate. These factors may be: incomplete administration of the oral dose; incomplete absorption from the alimentary tract, both of which could be avoided by giving the dose intravenously; retention in the tissues or body fluids; impaired renal function or incomplete collection of urine. However, if the excretion in twenty-four hours is found to be above 80%, it indicates that these interfering factors play a minor role and that the calculated production rate represents more than 80% of the true value.

Lazarus (1962) noted another drawback to the urine method. He used H^3-labelled/

H^3-labelled cortisol and proved that an important factor influencing the accuracy of this method was the rate of secretion on the day before the test; 70% of the metabolites measured are excreted on the day of the test and 30% on the day ~~before~~ after. Under basal conditions this factor will not significantly affect the results, but in non-steady states such as in Cushing's syndrome, after ACTH stimulation, and in stress the calculations may not be equal to the amount of cortisol secreted on the day of the test. The figures given by this method by Cope and Black for normal subjects are 4.9-25.3 mg./24 hrs.

In the blood method, the curve of decline of specific activities consist of two parts; an initial rapid decline after injection of the dose lasting about 30 minutes followed by a less sharp decline. Peterson (1959 a) interpreted this curve as indicating a fairly rapid distribution of cortisol into about 6 litres volume, and assumed that this volume was a single compartment in which the hormone has a metabolic half clearance time of about 80 minutes. Tait et al (1961), however, interpreted this curve as representing a two compartment model indicating an extremely rapid distribution into a space of about 6 litres (inner pool), followed by a relatively slow movement into a total volume of about 13 litres (inner and outer pools). The smaller volume can be regarded as a single compartment which has a metabolic half clearance time of about 30 minutes, and they worked out an equation for calculating the production rate from a double equilibrium model:-

$$\text{cortisol production rate} = \frac{R}{\frac{A}{\alpha} + \frac{B}{\beta}}$$

where/

where R = total radioactivity of the dose (cpm)

B = intercept of second part of the curve on ordinate (cpm/µg)

β = turnover rate as calculated from the second part of curve (pool/day)

A = intercept of corrected first part of the curve on ordinate (cpm/µg)

α = turnover rate as calculated from the corrected first part of curve (pool/day)

If a plot is made of the total radioactivity in a known volume of plasma as a function of time, a similar graph results from which the volume of distribution can be worked out. Dividing the total radioactivity injected by the figure of the intercept of the second part of the curve on the ordinate provides the volume of the inner pool. Dividing the same figure by the figure of the intercept of the corrected first part of the curve on the ordinate provides the volume of the outer pool.

Nugent et al (1961), using infusions of large amounts of non-radioactive cortisol to normal subjects receiving suppressive doses of dexamethasone, examined the levels of plasma corticoids during and after cessation of the infusion. Their observed data, compared with the predictions of the single-compartment-exponential disposal theory showed a poor correlation; corticosteroids did not disappear from the plasma as though they were in a single compartment and disposed of at a rate proportional to their concentration. They concluded that methods for quantitative measurement of adrenocortical function that utilise the assumption of single - compartment distribution and exponential disposal of plasma corticosteroids are based on a theory which does not describe the data accurately.

Tait (1963) using radioactive techniques showed, however, that unlike estimations/

estimations of aldosterone production rate, which necessitated using the double compartment model (because of the initial distribution in a volume of 20-30 litres), using a single compartment model for cortisol production rate (in spite of the presence of two exponentials), the resulting error is likely to be only about 10% when cortisol concentrations are low and when the volumes of distribution are small. The difference between the behaviour of cortisol and aldosterone may be due to a slower metabolism caused by the marked protein-binding of the former. Suggestions favouring a single mathematical approximation are; firstly, the possibility that there is a single rate-limiting step in the degradation of cortisol (Tomkins, 1959) and, secondly, the seemingly satisfactory agreement of plasma corticosteroids disposal data with first order kinetics when the logarithm of concentration is plotted as a function of time (Samuels et al, 1957; Peterson, 1959 b). However, an accurate mathematical description of plasma cortisol distribution and disposal is complicated by:

1. The number of corticosteroids with the dihydroxyacetone group (DeMoor et al, 1960 a); this can be avoided by complete separation of cortisol.
2. The limitation of diffusion into tissues by plasma-protein binding of cortisol.
3. The differences in the apparent volume of distribution and rate of disposal of cortisol when determined after tracer and load doses of cortisol.
4. The time necessary to ensure equilibration of cortisol within the body fluids (Samuels et al, 1957; Peterson et al, 1960).

The radioactivity counting technique used by Peterson and Wyngaarden (1956) utilised the gas-flow type of counter, while Cope and Black (1958 a) used/

used an end-window Geiger-Muller counter. In this study, cortisol production rate was estimated by both the blood and the urine methods but using a liquid scintillation counter. Glasstone (1958) pointed out that organic scintillators, either in the solid state or in solution, produce scintillations when exposed to nuclear radiation and that they are transparent to the light they emit. As a result, relatively large quantities of scintillator can be used; this ensures a high probability of interaction with the radiation and a high detection efficiency, especially when used with the sensitive photomultiplier tube. Glasstone enumerated the advantages of the scintillation counters which made them superior to other type of counters. These include their simplicity, great flexibility and versatility, high sensitivity to all forms of nuclear radiation, rapid response, very short resolving time, great efficiency and accuracy.

Adrenocortical stimulation and suppression tests

Making use of the discovery of the negative feed-back mechanism, several workers demonstrated the effect of administering exogenous ACTH as a means of assessing the maximum capacity of the adrenal cortex to secrete corticosteroids. Likewise, in the presence of an efficient negative feed-back mechanism, the endogenous pituitary ACTH should be suppressed by administration of synthetic glucocorticoids and this, in turn, should lead to suppression of endogenous corticosteroid secretion by the adrenal cortex. These are the bases of the adrenocortical stimulation and suppression tests widely used nowadays as complementary to the study of the adrenocortical function.

1. Adrenocortical stimulation tests

The response to ACTH affords a direct method for the assessment of adrenocortical capacity. It seems that the first of these tests was described by Thorn and co-workers in 1948 and was used in investigating adrenocortical insufficiency. It was called 'the four-hour intramuscular ACTH test', or "Thorn test". A preliminary eosinophil count was performed, then 25 I.U. of ACTH were injected intramuscularly, and four hours later a second eosinophil count was made. Normally, a moderate fall in eosinophil count occurs spontaneously due to the diurnal rhythm, but a fall of 50% or more excludes significant adrenocortical insufficiency. This test, however, was not quantitative, and the absence of the fall did not prove the diagnosis since some conditions, like acute allergy, may lead to increased production of eosinophils in the bone marrow exceeding the eosinopenic action of cortisol.

The second type of stimulation tests introduced was called 'the eight-hour intravenous/

intravenous ACTH test' (Renold et al, 1952) later called "the standard intravenous ACTH test". In this test 25 I.U. of ACTH in 500 ml. saline were infused within a period of exactly eight hours. Eosinophil counts and levels of 17-KGS or P-S chromogens in 24 hour collections of urine were made before and on the day of the test. Again, in this test, eosinophil count normally drop in eight hours by 94%; a drop of less than 50% is diagnostic of adrenocortical insufficiency. Also, a rise of 300-500% of urinary corticosteroids in 24 hours occurs in normal subjects; a rise of less than 100% is diagnostic of adrenocortical insufficiency. This test is sensitive, specific, and estimates the 'actual functional cortisol reserve' of the adrenal cortices and, when repeated over a period of two or three days in succession, measures the 'potential functional reserve', i.e. the maximal cortical production under exceptional conditions.

The eight-hour test produces a threefold to fivefold increase of corticosteroid output above the control levels, while a twenty-four hour infusion yields as much as tenfold increase which is a true absolute maximum. If the eight-hour test is repeated for several days, it could differentiate primary adrenocortical insufficiency, where no rise or a slight fall of the urinary corticosteroid output occurs after the first day, from hypopituitarism where gradual and sustained increase occurs after the first day. The intravenous ACTH test was modified and estimations of plasma corticosteroids were made before and after the test to avoid the need of accurate urine collection and to save time. Thus, 10 I.U. over a period of two hours, 25 I.U. over a period of 4 hours, 25 I.U. over a period of 6 hours (Eik-Nes et al, 1954 a) were proposed. Elevation of plasma corticosteroids excludes adrenocortical insufficiency and a hyper-response suggests Cushing's syndrome (Jailer/

(Jailer et al, 1954; DiRaimondo et al, 1958). Marked responsiveness suggests bilateral adrenocortical hyperplasia, a lesser degree of responsiveness suggests adrenal adenoma while cases of adrenal carcinoma are less responsive or even unresponsive (Lindsay et al, 1956; Nabarro et al, 1958; Birke et al, 1960).

Intravenous ACTH administration, however, is sometimes risky. Sudden elevation of blood pressure may occur and is sometimes fatal. To render this test simpler and safer, ACTH-gel was used (DeFilippis and Young, 1957) but it was, however, applied differently by various authors:

1. Bayliss and Steinbeck (1954) administered 80 I.U. once and estimated plasma 17-hydroxycorticosteroids after 4, 8 and 24 hours.
2. Dyrenfurth et al (1960) administered 20 I.U. every 6 hours for 48 hours and they estimated urinary and plasma steroids during and after the stimulation.
3. Jenkins (1961) administered 40 I.U. and estimated plasma corticoids after two hours; 'two-hour test'.
4. Futterweit et al (1962) administered 40 I.U. and estimated plasma corticoids after 4 and 6 hours.
5. Brooks et al (1963) administered 20 I.U. twice daily for 4 days.

The standard intravenous test, however, is still widely used (Birke et al, 1960; Hoet et al, 1961; Silverman et al, 1963; Schteingart et al, 1963). In this thesis stimulation tests were studied using ACTH-gel.

2. <u>Endogenous - ACTH suppression tests</u>

Suppression of urinary 17-KS output by cortisone has been known for a long time. It was Laidlow et al (1955) who applied 9 α-fluorocortisol (9α-FF) (3 mg./12 hours/

(3 mg./12 hours for 4 days and 25 mg./day for 3 days) to patients with Cushing's syndrome, and failed to demonstrate a significant decrease in either 17-KS or 17-KGS. Cope and Harrison (1955) and Cope (1956) on the other hand showed that 9α-FF in divided doses totalling 5-10 mg. per day partially suppresses urinary corticosteroid excretion of cases of Cushing's syndrome due to adrenal hyperplasia, but did not suppress patients with carcinoma. Gennes et al (1956) using 9α-FF in doses of 2-4 mg./day found that urinary steroid excretion was abnormally resistive to suppression in cases of Cushing's syndrome due to adrenal hyperplasia but definite suppression occurred when the dose was increased to 6 mg./day. Jenkins and Spence (1957) also noticed this partial suppression of urinary corticosteroids in Cushing's syndrome using 9α-FF in doses of 1 or 2.5 mg./6 hours for 2-3 days. Nabarro and his associates (1958), on the other hand, noticed that this partial suppression was demonstrated only in 4 out of 9 cases of adrenocortical hyperplasia, but failed to demonstrate suppression in two patients with adrenal tumour. They used 9α-FF in doses of 10-20 mg./day.

Liddle (1960) suggested that, when performed under carefully controlled conditions, the ACTH-suppression tests can be reliable diagnostic procedures. He proposed his tests which have been extensively used in the study of the pituitary-adrenocortical inter-relationship. 9α-FF and, better still, its extremely potent C-16-methyl derivative dexamethasone were the two synthetic glucocorticoids used. Administration of 0.5 mg. every 6 hours for two days followed by 2.0 mg. every six hours for another two days were said not only/

only to differentiate normal subjects (who will suppress markedly by the smaller dose) from cases of Cushing's syndrome, but also to differentiate cases of adrenocortical hyperplasia, in whom relatively more suppression occurs especially with the large dose, from cases of adrenocortical adenoma or carcinoma, who are more resistent to suppression even to the large dose (especially cases of carcinoma which on the whole do not suppress at all). Suppression tests as described by Liddle were widely used, mostly with encouraging results (Liddle and Williams 1962; Slater et al, 1962; Futterweit et al, 1962; Gogate and Prunty, 1963; Schteingart et al, 1963) but sometimes with disappointing results (Dyrenfurth et al, 1960; Meador et al, 1962; Silverman et al, 1963; Baird, 1963). In this thesis suppression tests using dexamethasone were also studied.

3. Specific inhibition of adrenocortical enzymes

It is known that hydroxylase enzymes are necessary for the biosynthesis of cortisol by the adrenal cortex. Blocking of the action of these enzymes can occur in certain disease conditions, such as in congenital adrenal hyperplasia, with the result that cortisol secretion is diminished and its precursors and their metabolites like THS and pregnanetriol are excreted in urine in large quantities.

Recently, synthetic compounds have been introduced whose action is to block certain hydroxylase enzymes specifically. This leads to reduction of cortisol synthesis and accordingly, through the negative feed-back mechanism, in an increase in ACTH production and secretion, which in turn will enhance the biosynthetic pathways reflected as increased excretion of precursors of cortisol and their metabolites in urine. SU-4885 (Metopirone), an 11β-hydroxylase/

hydroxylase inhibitor, is the most important of these compounds and its use is a measure of the so called "Pituitary-ACTH reserve" or the "Pituitary-Adrenal reserve". Liddle et al (1958; 1959) applied this test to study the intermediate metabolism of corticosteroids and as a diagnostic tool in adrenocortical dysfunctions. The use of this test, however, appears to be more limited in the diagnosis of cases of congenital adrenocortical hyperplasia (Cleveland et al, 1962; Liddle and Island, 1962; Grant, 1962).

Complementary tests of adrenocortical function

These are indirect tests which are carried out whenever adrenocortical dysfunction is suspected. They are suggestive or confirmatory rather than diagnostic.

Estimation of sodium, potassium, chloride and bicarbonate in serum: elevated sodium levels and lowered potassium and chloride and sometimes hypokalaemic alkalosis occur with excess adrenocortical activity. These changes are reflected, and can also be detected in the saliva.

Fasting blood sugar, glucose tolerance curve and glucose in urine: elevated fasting level and diabetic curve with glycosuria are common with excess cortisol secretion.

Blood lipids and cholesterol are also raised with excess cortisol secretion.

Blood picture. Relatively low counts of lymphocytes, eosinophils and basophils with relative and absolute neutrophilia, leucocytosis, polycythaemia, increased packed cell volume and platelet count are frequent with cortisol excess.

X-ray of bones may reveal ostoporosis, compression fractures of vertbrae, fractured ribs or dorsal curvature of spine often observed with hypercortisol states.

X-ray of the skull may reveal enlarged sella turcica denoting a pituitary tumour as a cause of the hyperfunction of the adrenal cortex.

X-ray of the chest may reveal cardiac enlargement as a result of raised blood pressure often observed in cases of hypercortisol secretion.

Electrocardiographic tracings often reveal low voltage and prolonged P-R and Q-T intervals with cortisol deficiency states.

Urine/

Urine examination may reveal polyuria and increased calcium excretion in hypersecretion states of the adrenal cortex.

ACTH assays in plasma or urine can be elevated in adrenocortical insufficiency and in hyperfunction due to ACTH-dependent hyperadrenocorticism.

Chapter III: Cushing's syndrome and obesity

Cushing's syndrome

Definition and aetiology

In pathological conditions of the adrenal cortex, namely hyperplasia, adenoma or carcinoma, a variety of syndromes may appear depending upon the excess production of one or the other of glucocorticoids, mineralocorticoids, androgens or estrogens. Cushing's syndrome is the syndrome of preponderant glucocorticoid excess, properly called "the syndrome of excess cortisol".

Pure forms of the disease are not common and usually there are varying degrees of excess secretion of one or more of the other hormones as well. This syndrome was first described by Harvey Cushing (1932) who suggested that basophilic pituitary adenoma was the cause behind the bilateral adrenocortical hyperplasia leading to the clinical manifestations of the syndrome. As much as 15% of normal subjects, however, show small basophilic adenomata of the pituitary at autopsy without previous history suggesting adrenocortical hyperfunction. The aetiology of the syndrome is still obscure. The most accepted theory is that it is due to excess corticotropin production by the anterior pituitary, but in the absence of the anterior hypophysis, the secretion of cortisol is greatly reduced but interestingly enough not to zero. The possibilities that exist as to the mechanism by which Cushing's syndrome may be brought about include (1) stimulation from the hypothalamus via the pituitary to the adrenal cortex with adrenal hyperplasia; (2) hypothalamic stimulation plus pituitary tumour; (3) autonomous pituitary tumour; (4) adrenal hyper-reactivity due to inherent adrenal abnormality as well as the known and (5) autonomous adrenal cortical tumours. Recently McCullagh (1962) and Forsham (1962) extensively reviewed this clinical condition.

Incidence and pathogenesis/

Incidence and pathogenesis

Cushing's syndrome is a rare condition occurring nearly once in every thousand persons and 4-5 times commoner in females. It occurs in any age but mostly in the third and fourth decades. 60% of the cases show bilateral adrenocortical hyperplasia, 30% show adrenocortical tumours, adenoma or carcinoma, and 10% have normal adrenal glands. In bilateral adrenocortical hyperplasia the microscopic picture is not unlike that observed after stimulation with ACTH, with widening of the cortex. Pituitary tumours are rare and enlargement of the sella turcica is even rarer.

A fascinating feature of Cushing's syndrome is its occasional appearance in primary malignant disease in various organs. This includes; bronchogenic carcinoma, ovarian neoplasms, thymic, pancreatic, prostatic and thyroid carcinomata (McCullagh, 1962). It has also been recently reported in association with phaeochromocytome (Williams et al, 1960).

Clinical manifestations

a. Signs and symptoms due to excess cortisol

1. Obesity is present in 97% of cases. There is not necessarily any marked gain in weight; obesity is mainly due to redistribution of fat, with a particular tendency for a great proportion of fat to be deposited along the trunk, and especially on the anterior abdominal wall, around the shoulder girdle, in the supraclavicular region, in the upper dorsal region (buffalo hump) in the neck and in the cheeks (moon face and fish mouth appearance) but characteristically sparing the extremities, which appear unduly thin. The moon face, trunk obesity with thin extremities give the "spider appearance" characteristic of Cushing's syndrome. The mechanism by which these changes are/

are produced is not yet known.

2. In 75% of cases thinning of the skin due to wasting of skin muscles and the disappearance of elastic fibres occurs leading to subcutaneous dehiscence and purple striae over the abdomen, thighs and upper arms forming deep gullies in the skin which can be felt by the fingers. Red striae, however, are not restricted to Cushing's syndrome; they may be present also in pregnancy and in rapidly growing and developing girls, in whom they may radiate from the areola like spokes on a wheel. There is no excess corticosteroid in these cases to account for them; it is merely excessive stretching of skin with consequent dehiscence of the subcutaneous tissue. Gogate and Prunty (1963), however, reported that the striae in subjects with Cushing's syndrome are characteristically broad, of purplish colour, have an atrophic dermatitis and occur mostly on the flanks and lower abdominal wall. This is in contrast to striae sometimes found in obese subjects which are neither purple nor associated with thinning of the skin; their colour is of a typical light pink nature. These workers found that striae in obese subjects occur on the flanks, abdomen and about the axillae, upper arms and breast and occasionally on the buttocks. They also observed that, in a given area, striae in obese subjects are more numerous than in Cushing's syndrome. The skin in Cushing's syndrome is of fine texture, almost paper like. The face is plethoric because of transparency of subcutaneous tissue and polycythaemia. There is easy bruisability with ecchymoses (in the presence of normal or elevated platelet count) and poorly healing wounds, frequently infected.

3. General and progressive muscle wasting associated with extreme weakness and/

and negative nitrogen balance. This may be mainly due to potassium depletion and to the anti-anabolic effect of cortisol.

4. Thinning of the bones (demineralisation) and marked osteoporosis, due to the anti-anabolic effect of cortisol, resulting in compression fractures of the vertebrae, kyphosis, fractures of ribs and commonly associated with backache occur in Cushing's syndrome. Increased excretion of calcium in urine may occur and nephrosclerosis or urinary calcinosis are not uncommon.

5. Marked and irreversible progressive atherosclerosis in the large blood vessels with systolic and, occasionally later with diastolic hypertension occur in about 90% of cases.

6. Hyperglycaemia, glycosuria and diabetic glucose tolerance curve. Latent diabetes occurs in 80% of subjects and overt diabetes in 20%.

7. Sodium retention, low serum potassium and chloride. Hypokalaemic hypo-chloraemic alkalosis is seen in about 15% of cases.

8. Depression, psychosis and mania are frequent.

b. Signs and symptoms due to excess androgen secretion or excess breakdown of cortisol or both

These include acne; bushy eyebrows; wiry, thin and lateral receding scalp hair; hirsutism; marked increase in musculature in females with changes to the male figure; metrorrhagia or amenorrhoea in females; impotence in males; deepening of voice and clitoral enlargement in females. Only the latter symptom can be attributed to androgens since all the others occur also with excessive administration of cortisol.

c. Signs and symptoms due to excessive secretion of mineralocorticoids and/or cortisol secretion

These/

These include hypertension; hypokalaemia; hypernatraemia; polyuria not responding to antidiuretic hormone (nephrogenic diabetes incipidus) which may be due to potassium depletion nephropathy; oedema due to diminished glomerular filtration rate and increased plasma volume.

Clinical manifestations due to excess estrogens are rarely encountered.

Course and diagnosis

The onset of the syndrome is insidious and the course is progressive with transitory remissions and exacerbations often associated with increased psychic stress. Patients become bed-ridden due to muscle weakness, multiple fractures or intercurrent infection. If untreated, patients die within five years of general debility, vascular accidents, infection or diabetic coma. The more the clinical manifestations of the syndrome are present together, the more it should be suspected. Sudden onset may denote adenoma or carcinoma. Even when all the signs and symptoms associated with Cushing's syndrome are present, diagnosis, however, still needs to be confirmed and the extent and nature of the adrenal hyperactivity evaluated by laboratory tests.

From the discussions in Chapter II measurement of urinary 17-KS excretion is of little help in the diagnosis of this condition. On the other hand, elevated plasma corticosteroid or cortisol level with loss of the normal diurnal variation, elevated urinary Porter-Silber chromogens and 17-KGS especially in the mixed type of the syndrome, are diagnostic. However, sometimes the results of these tests overlap with results in normals and in obese subjects and the blood estimations are liable to variation with emotional upset. Moreover, day-to-day fluctuation of cortisol secretion is characteristic of this syndrome which further adds to the liability of obtaining normal results with/

with these tests. About 90% of cases, however, could be diagnosed by these tests, especially if repeated.

More sensitive tests are:estimations of free cortisol in urine, cortisol production rate by radioactive dilution techniques, plasma non-protein-bound cortisol, tests of adrenocortical capacity by stimulation with exogenous ACTH, and tests of pituitary-adrenocortical suppressibility by dexamethasone. These tests are less simple and need special training; moreover they are time consuming and are only suitable for research work. Reliable routine tests are unfortunately still unavailable. The last mentioned two tests may also be valuable in differentiating bilateral hyperplasia from tumours. The laboratory diagnosis could be supported by the X-ray findings of osteoporosis (thin skull, cod fish vertebrae due to compression of bodies of vertebrae by the nucleus pulposus), elevated ACTH assays in blood or urine, the haematologic findings of lymphopenia (less than 15%) eosinopenia with relative and absolute neutrophil leucucytosis, total white cell count of 10,000 - 20,000 in the absence of demonstrable infection, polycythaemia and packed cell volume of 50% of more, increased platelet count, as well as by elevated fasting blood sugar level, glycosuria, diabetic glucose tolerance curve and by the finding of hypokalaemic hypochloraemic alkalosis. Diagnosis of tumours can be confirmed by plain X-ray, tomography, intravenous pyelography, aortography, arteriography, retroperitoneal pneumography and X-ray of the sella turcica.

<u>Treatment</u>

a. <u>Bilateral adrenocortical hyperplasia</u>

Because cortisol is supposed to be the responsible direct agent causing this/

this clinical condition, the rational step is to reduce or eliminate this factor by one of the following methods;

1. Unilateral adrenalectomy. With this line of treatment there is a 30% chance of a complete return of the pre-operative steroidogenic capacity.

2. Subtotal adrenalectomy. With this approach, there is a possibility of leaving too much adrenal tissue and accordingly of recurrence of the syndrome or of leaving too little with the inevitable development of Addison's disease unless replacement treatment is given.

3. Bilateral adrenalectomy. Again, with this line of treatment there is subsequent need for continued substitution therapy.

4. Electrocoagulation of the pituitary, separation of the pituitary stalk, partial or total hypophysectomy when Cushing's syndrome is due to pituitary adenoma. With these approaches sterility develops and the substitution therapy is more complex, viz. cortisol, desiccated thyroid and stilbosterol in females or testosterone in males.

5. X-ray therapy to the pituitary (5000 r), the use of proton beam or the implantation of radon, gold or yttrium-90 seeds. Again, with these lines of treatment there is a 30% chance of remission.

6. Combined unilateral adrenalectomy and pituitary irradiation. A 70% chance of remission exists with this approach.

7. Adrenocortical inhibitory therapy is still in its experimental stage and seems to be only applied in carcinomatous conditions or in cases where remissions might be anticipated as in patients with psychosis or hysterical anxiety states. This is achieved by administering either; amphenone; the insecticide/

insecticide DDD or its less toxic and more active derivative o,p'-DDD or the inhibitor of cholesterol biosynthesis triparanol (MER - 29). In all of these drugs toxic effects can be pronounced and tolerance eventually develops and it seems that their use is only limited to hopeless cases.

Bilateral adrenalectomy seems to be the therapy of choice in the rapid progression of Cushing's syndrome, especially when the syndrome is accompanied by psychosis, multiple bone fractures or severe uncontrollable diabetes.

b. Tumours

Treatment is by extirpation of the tumour or most often by the removal of the entire gland. Due to the fact that the contralateral gland will be atrophic, substitution therapy is most important. This must be gradually reduced to encourage the contralateral adrenal cortex to regain its function. A very few cases, however, require the substitution therapy permanently because of irreversible atrophy of the contralateral gland and/or the corticotropin-producing elements of the anterior pituitary gland.

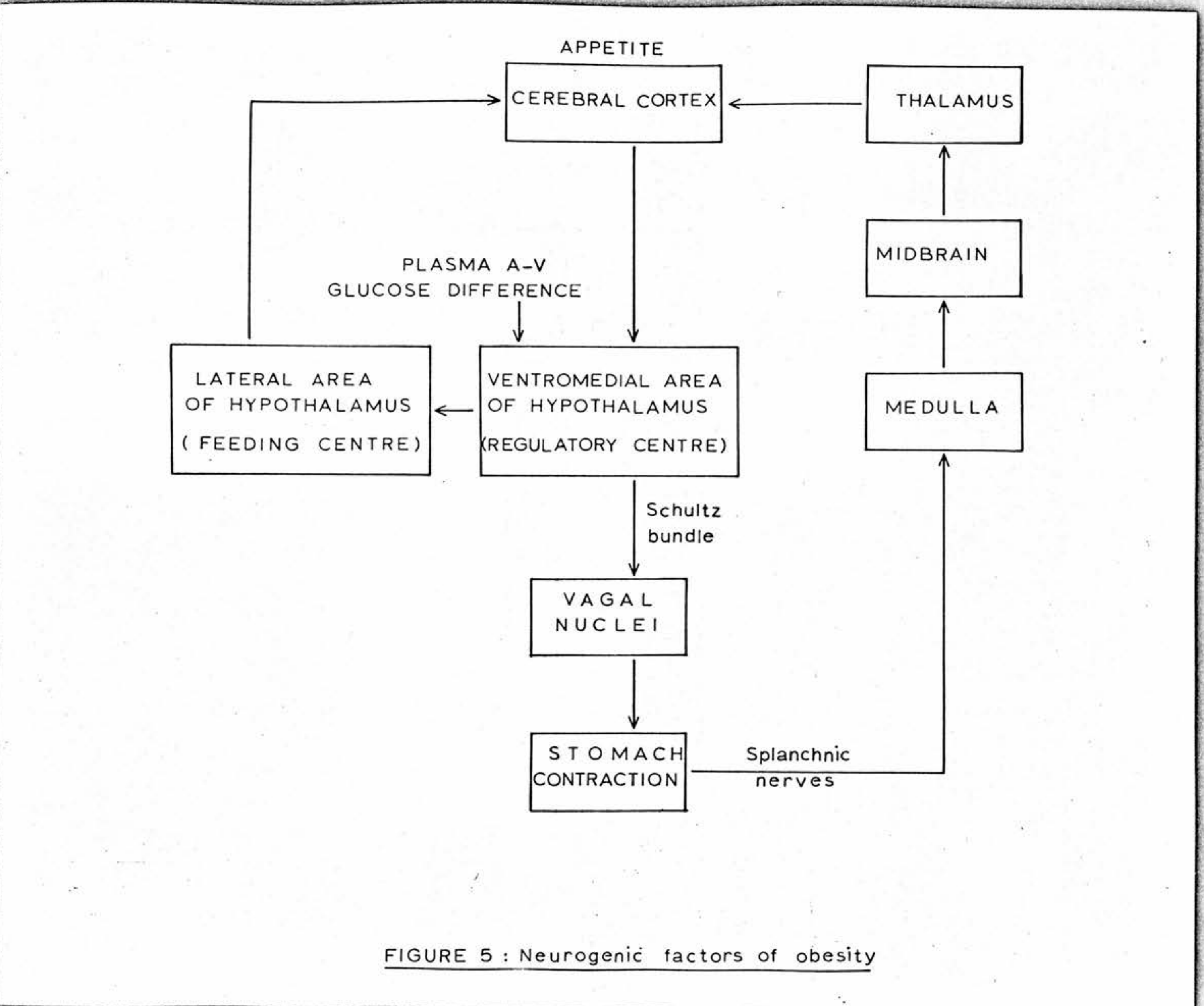


FIGURE 5 : Neurogenic factors of obesity

Obesity

Definition and aetiology

Obesity is the increase in weight of 15% or more above the 'desired weight', calculated according to the height and body build. Normally about 12% of the body weight in males and 19% in females occurs as fat depots. Thus, a man is obese when the total fat content of his body exceeds 25-30% of the total body weight, and a woman is obese when hers exceeds 30-35%. The subject of obesity was excellently reviewed recently by Williams and Glomset (1962).

Numerous factors contribute to development of obesity, in the same or different subjects, some of these initiate obesity and some of them are induced by obesity which they then intensify thus setting in motion a vicious circle. But once obesity has become marked, they all become operative, contributing to the number of disturbances. The work of Mayer (1959 a, 1959 b, 1960 a, 1960 b) has contributed much to the understanding of these various factors. These include:

1. Neurologic factors (see figure 5). Normally the ventromedial area of the hypothalamus, called 'the regulatory centre', acts as a glucostat. When hyperglycaemia occurs or there is an increase in arterial-venous glucose difference, impulses travel to the lateral hypothalamic area, called 'the feeding centre' inhibiting it. On the other hand when glucose utilization increases, hypoglycaemia leads to the removal of this inhibitory effect on the feeding centre. Impulses created by this mechanism as well as those coming from the cerebral cortex as a result of visual, olfactory, or other stimuli are transmitted through Schultz bundle, to the vagal nuclei, to the stomach stimulating/

stimulating contractions. Afferent impulses travel through the splanchnic nerves, medulla, midbrain, thalamus to the cerebral cortex. A sensation of hunger is produced in the subcortical area and a conscious desire for food is created in the cerebral cortex. Dysfunction of the regulatory centre, therefore, leads to removal of the inhibitory impulses and marked ingestion of food and obesity. Convincing evidence to the validity of this theory is available in cases of craniopharyngioma, meningitis and trauma to the hypothalamus in connection with surgical operations. But although many patients are obese, there are very few in whom the obesity is clearly the result of hypothalamic damage.

2. Psychological factors. 'Reactive obesity' is a well known condition where 'compulsive eaters', usually suffering from grief or severe depression, sometimes overeat in response to a compensation for tension and frustration. The more or less automatic food-regulatory mechanisms, which seem to work nicely at essentially a subconscious level in normal individuals, become blunted and are succeeded by the conscious effort to control food intake. Since the guide lines do not seem to be clearly demarcated, the subject ends up by ingesting far more food than he or she needs. It is also known that frontal lobe lesions can increase the appetite by influencing the hypothalamic food-intake centre, as well as the amount of exercise and the emotional reactions. But frontal lobe lesions are very rare in obese patients, and emotional disturbance and obesity may co-exist without necessarily being related to each other. Sometimes marked obesity can occur without significant psychologic disturbance, and sometimes marked psychologic problems similar

to/

to those found in the obese occur without obesity or even with weight loss. However, there are definite psychologic consequences of obesity; an already lazy, lethargic and weak-willed obese person may feel discriminated against in social life and may develop hostile attitudes, due to repeated reproaches by physicians, parents or well wishers. Obesity may be intensified leading to more withdrawal and avoiding physical or social activities, further intensifying the obesity and so on.

3. Endocrine and metabolic factors. Obesity is frequently associated with hypopituitarism, hyperpituitarism, hypothyroidism, hypogonadism and some pancreatic disorders (Spence, 1961). Grafting of ACTH-secreting pituitary tumours in animals leads to obesity. Also injection of glucocorticoids, and in Cushing's syndrome definite increase and redistribution in the fat depots are observed associated in some cases with increased plasma level or urinary excretion of corticosteroids. Castration in males after puberty leads to obesity and eunuchoid males are usually obese. Administration of testosterone tends to reduce and estrogens to increase obesity in males. The following conditions associated with endocrine and/or metabolic disturbances occur with increased frequency in obesity: diabetes, increased resistance to insulin, hypertension, nephrosclerosis, atherosclerosis, biliary calcinosis, hirsutism, amenorrhea and sterility.

4. Genetic factors. It is usually difficult to differentiate between genetic and environmental factors because of the eating habits of the family, the social and economic status, the emotional status, etc., all influence the familial /

familial incidence of obesity. It is interesting to know, however, that Mayer (1959 a, 1960 a) found that less than 10% of children were overweight when both parents were normal, 40-50% were overweight when one parent was obese and 80% overweight when both parents were obese, and that studies of identical and fraternal twins have revealed that family food habits are not the only or even main factor concerned in the development of obesity. When there is a distinct excess of food, there is an instinct to accumulate greater body stores than are needed, presumably a carry-over from earlier days when cycles of plenty and famine prompted the body to store extra energy while it was available. Perhaps this genetic factor can explain the two types of obesity, the easier to treat 'juvenile or constitutional' type of early development and the fluctuating and more difficult to treat 'adult or developmental' type starting later in life. Furthermore, the obese hyperglycaemic syndrome is suggested to be a genetic disorder of a Mendelian recessive type.

5. Extrinsic factors. In view of the high incidence of mild obesity, these are the most important factors leading to obesity. Social, economic and family factors, customs and habits of eating, type of processing of food, quality and quantity of food provided, type of occupation and readily available transportation, are all of great importance.

Incidence and pathogenesis

Obesity is a very frequent condition, and if the actual weight is compared to the ideal weight, as much as 40% of the total population of most developed countries can be considered obese.

When/

When lipid stores exceed their limits, new fat cells are formed to accommodate new fat deposition. Presumably macrophages and other elements of the reticuloendothelial system take over this role. Obesity is associated with enlargement of the gastrointestinal tract, liver, kidney and other organs, increased rate of intestinal absorption and decreased spontaneous physical activity. A suggested sequence of changes (Williams and Glomset, 1962) accounting for most, or all of the clinical manifestations of obesity is: ingestion of great quantities of food leads to stimulation of the β-cells of the pancreas producing more insulin. Excess insulin promotes lipogenesis. Insulin also increases the output of glucocorticoids, which in turn play an important role in stimulating increased production of insulin antagonists. At least some of these antagonists have been shown to antagonise the action of insulin in muscle but not in adipose tissue. The body is thus left in a state where there must be too much insulin action on adipose tissue for insulin action on other tissues to be sufficient. This possibly, then could account for a number of the so-called complications of obesity which simulate those of diabetes mellitus. With marked reduction in food intake these biochemical and clinical alterations are reversed in due course of time.

Clinical manifestations

In the majority of cases obesity constitutes only a relatively mild deviation from normal without significant signs and symptoms. But as the process becomes severe and of long duration we may find; (1) psychological disturbances; anxiety, depression, feeling of inferiority, etc., (2) heart affection;/

affection; dyspnea, orthopnea, cyanosis or frank heart failure and oedema, (3) decreased vital capacity; decrease in the gaseous exchange in the lungs with drowziness and other features of the 'pickwickian syndrome', (4) skin thickness, coloured striae, fungus and yeast infection, intertrigo, (5) hirsutism, (6) gastrointestinal disorders such as dyspepsia, liver cirrhosis, cholecystitis, cholelithiasis, (7) varicose veins of the legs, (8) hypertrophic arthritis, (9) menstrual abnormalities; amenorrhoea, oligomenorrhea and sterility, (10) hyperglycaemia, glycosuria and decreased glucose tolerance, (11) atherosclerosis, coronary occlusion, hypertension, (12) nephrosclerosis and (13) increased incidence of malignancies (Williams and Glomset, 1962). There is a distinct increase in morbidity and mortality with increasing degrees of obesity. Obesity is not quite as harmful in women.

Course and diagnosis

With significant weight reduction, associated conditions likewise improve. This applies only to mild obesity. Obesity is much easier to deal with in its early phases when there are fewer psychological, metabolic and endocrine alterations. With severe obesity, the sensitivity of the satiation centre seems to be greatly blunted, so that the normal food - regulating mechanisms do not prevail. Obesity, however, tends to produce hyperinsulinism and hyperadrenocorticism which can in turn lead to further the obesity. Thus moderate and severe obesity is usually progressive, irreversible and very difficult to treat.

Assessment of the degree of obesity can be made by one of several methods; inspection (correlating muscular development with height and skeletal build); weight as correlated to height, best and easily found out from tables constructed to/

to indicate the 'desirable' weights for men and women according to their heights and frames (see appendix III); measurement of the thickness of subcutaneous tissue either by the pinch technique, by calipers or by roentgenograms of the soft tissue; densimetric determinations by weight in air and under water; or by estimating the total body fat which is the difference between the lean body mass (estimated by heavy water dilution technique) and the total body weight.

Treatment

As it is easier to maintain normal weight than to reduce excess weight, preventive measures are most important in the form of educational programmes for the general public and for those who supply and deal with food. Individual psychological problems of obese patients should be evaluated and treated and patients warned that during weight loss, some psychological problems may be intensified and fresh ones acquired.

Attention should be given to the caloric equivalents of different food elements of diet. Carbohydrates and proteins tend to promote retention of approximately 3 parts of water per each part of these food materials that is stored. Fat is deposited without a store of any significant amount of water. Thus, with the loss of a pound of protein there is a loss of four pounds of body water. When the protein intake is increased, the lost weight is rapidly regained. Thus, although the weight loss may appear greater with a low-protein diet, true weight loss is best accomplished with a diet high in proteins and low in fat and carbohydrates (of 800 - 1200 calories) and rich in vitamins and minerals, i.e. vegetables and fruits of low -carbohydrate value (see appendix VI).

Anorexigenic/

Anorexigenic drugs; amphetamines, may be of help especially in the early phases of weight reduction, propping up the patient's will power until progress begins after which the patient carries on under his own will. These drugs, however, are not free from side-reactions and eventually become of limited value because tolerance develops. Various pituitary and thyroid hormone products as well as nauseating and diuretic drugs are useless.

Exercise and massage are adjunct to weight reduction.

Aim, Subjects and outline of this study

The previous review of Cushing's syndrome and obesity, shows very clearly that many clinical manifestations and laboratory findings can occur in either of the two conditions, such as; hypertension, psychological changes, hyperglycaemia, impaired glucose tolerance, hirsutism, cutaneous striae, infective conditions, oedema, raised plasma level and urinary excretion of corticosteroids, etc. But it is also clear that prognosis and the therapeutic approach are so different; in Cushing's syndrome the patient if not treated dies within five years (Forsham, 1962) and the treatment of choice is adrenalectomy while obesity is a less grave condition which is treated mainly by dietary regimen. This, together with the fact that a previous study in this department (deWitt, 1961) failed to separate clearly normal subjects from patients suffering from Cushing's syndrome on the basis of urinary excretion of 17-KS, 17-KGS or even cortisol production rate by either the urinary or blood method, provided the initiative for this study. The aim of this study was to try, by careful grouping of the subjects and by selective application of certain tests of adrenocortical function, to separate clearly patients suffering from Cushing's syndrome from simple obese and from normal subjects.

Accordingly, the subjects of this study were divided into four groups:

1. Normal subjects; who were either normal healthy volunteers, who showed no symptoms or signs of disease; or 'hospital normals' who were either patients suffering from minor clinical conditions such as bronchitis, dermatitis, dyspepsia, etc., patients waiting to be operated upon for minor surgery, e.g. piles/

of ACTH to allow pituitary-adrenocortical axis to get back to its normal state. In addition, from the technical point of view, not more than two cases could be studied at the same time. This, together with the fact that it was very difficult to find volunteers to provide for the control group and that cases of Cushing's syndrome were very rare, accounted for the relatively small number of cases studied especially among the normal and Cushing's groups.

Each case in this study was investigated first clinically by recording the presenting symptoms and their duration, family history of diabetes, obesity or endocrine disease, assessment of the degree and distribution of obesity if present followed by a thorough clinical examination. Then a series of chemical tests were carried out; to assess the adrenocortical function under basal conditions and in response to stimulation with exogenous ACTH and to endogenous-corticotropin suppression by dexamethasone administration. Other chemical tests were performed to exclude renal insufficiency; to exclude liver insufficiency; to detect diabetes; to detect any electrolyte disturbance or alkalosis and to find out the relationship between adrenocortical function and both the total body muscle mass and the total body fat. Other laboratory investigations were performed; these included X-ray of the chest to detect any cardiac enlargement; X-ray of the skeleton to detect osteoporosis; haematologic investigation; electrocardiography; medical photography; and histological examination of the removed glands in cases of Cushing's syndrome treated by adrenalectomy.

A special proforma was designed for this research. All the information available from the clinical and laboratory investigations of each case could be/

be recorded in a copy of this proforma, thus facilitating study and interpretation of the data in each case.

It was planned that treatment of cases of Cushing's syndrome should be either by bilateral adrenalectomy followed by substitution therapy or by bilateral adrenalectomy with transplantation of a part of one adrenal into the rectus sheath of the anterior abdominal wall. Obese subjects and 'Cushingoid' subjects in whom the suspicion of Cushing's syndrome was not so strong as to warrant surgery were treated by a low caloric dietary regimen. It was also planned to repeat the tests of adrenocortical function in obese subjects and 'Cushingoid' subjects treated by dieting if they responded sufficiently to the dietary regimen, (see appendix IV) by a significant reduction in weight, to see if there was any correlation between the intensity of obesity and the state of adrenocortical function. It was planned also to repeat these tests in cases of Cushing's syndrome treated by transplantation to assess the success of such operation, and in 'Cushingoid' patients in whom mild clinical manifestations became florid to see if there was a correlation between the intensity of the clinical condition and the state of adrenocortical function.

Part II

Methodology

A. Chemical methods

1. Estimation of cortisol production rate

Cortisol production rates were estimated by both blood and urine methods on three occasions in each patient (1) a first basal estimation, (2) a second estimation a week later following ACTH stimulation, and (3) a third estimation, two weeks after the second one, following dexamethasone suppression.

In each estimation 1 μc. 4-C^{14}-cortisol was given intravenously at 9:30 a.m. Later on, however, with the aim of investigating the problem of plasma protein binding to cortisol it proved more convenient to increase the dose to 2 μc.

i. Estimation of cortisol production rate by blood method

The method was based largely on that described by Peterson and Wyngaarden (1956).

Principle (see introduction, Chapter II)

Reagents

1. Cortisol standard
   (a) Stock standard: 0.25 mg. cortisol (B.D.H.)/ml. absolute ethanol.
   (b) Dilute stock standard: 2 μg. cortisol/ml. absolute ethanol.
   (c) Working standard: 0.2 μg. cortisol/ml. absolute ethanol.
2. Cortisol marker (for chromatography): 2 mg. cortisol/ml. absolute ethanol.
   All cortisol standards and marker, were kept at 4°C.
3. Dichloro-methane (Methylene chloride) (B.D.H.) Analar grade.
4. Benzene (B.D.H.) Analar grade.
5. Sodium hydroxide (B.D.H.) 0.1 N approximately.

6./

6. Acetic Acid (M & B) 0.1 N approximately.

7. Sulphuric acid (Hopkins & Williams) Analar grade. 80% (v/v) in distilled water.

8. Purified ethanol: 40 pellets of potassium hydroxide (B.D.H.) were added to about 500 ml. absolute ethanol, refluxed for two hours, and on gentle distillation the first and last portions were discarded and the middle one was collected.

9. Radioactive compounds: Two types of radioactive cortisol were used in this study:-

(a) $4\text{-}C^{14}$-cortisol of specific activity 25 millicuries per millimole was obtained from New England Nuclear Corp. Boston, Massachusetts, U.S.A. This substance was contained in vials each containing either 10 or 50 µc. in 1 ml. 10% methanol/benzene.

The contents of the vial were carefully transferred by a clean sterile 2 ml. syringe to a Quickfit (C24, 50 ml.) test tube. The vial was rinsed several times with 1 ml. absolute ethanol and the rinsings added to the test tube containing the radioactive material. This solution of radioactive cortisol in ethanol was then evaporated to dryness under reduced pressure using a water pump and a water bath at $40^{\circ}C$.

The molecular weight of cortisol is 362. Its specific activity was 25 millicuries/millimole ($\equiv$ 25,000 µc./m.mol.) so that the specific activity was $\frac{25,000}{362}$ = 69.1 µc./mg. The cortisol content of the vial was 725 µg., but since it was convenient to use cortisol whose specific activity was about 20 µc./mg. 1.81 mg. non-radioactive cortisol was added.

To the dry residue, 7.25 ml. of cortisol standard (0.25 mg./ml.) were added,/

added, together with 5.25 ml. absolute alcohol. The tube was stoppered and left overnight in the deep freeze. Next day 37.5 ml. sterile distilled water were added. The total volume was 50 ml. containing 50 µc. in 25% absolute alcohol. 1 ml. of this solution containing 1 µc. served as one dose.

To evaluate the specific activity of the dose solution, 0.1 ml. of this solution was diluted with absolute alcohol to 17.5 ml. 1 ml. of this dilute dose solution was used for the estimation of radioactivity and 1 ml. for the estimation of cortisol content. The specific activity of the dose solution was calculated from the formula:

$$\text{S.A. dose} = \frac{\text{Radioactivity in 1 ml. dilute dose (cpm)}}{\text{Cortisol in 1 ml. dilute dose } (\mu g)} \quad \text{cpm}/\mu g.$$

(b) $4\text{-}C^{14}$-cortisol of specific activity 22.3 millicuries/millimole was obtained from the Radiochemical Centre, Amersham, Buckinghamchire, England in sealed ampoules containing 50 µc. in 1 ml. of a benzene:ethanol (9:1) mixture.

The ampoule was carefully cut with a file and the contents transferred to a test tube and evaporated as described before. It was thought better at this stage, however, not to add any cortisol standard to the test tube in order to minimise the amount of cortisol injected lest this should introduce an error into measurement of cortisol production in patients with markedly suppressed plasma cortisol levels due to the administration of dexamethasone. The specific activity in this case was $\frac{22,300}{362} = 61.7$ µc./mg.

To the dry residue 5 ml. of absolute alcohol were added and the volume made up to 25 ml. with distilled water. 1 ml. of this solution served as one/

one dose and contained 2 μc.

Dilute dose solution: 0.1 ml. of the dose solution was diluted with absolute alcohol to 35 ml. 1 ml. of this dilute dose solution was used for the estimation of radioactivity and 1 ml. for the estimation of cortisol content from which the specific activity of the dose solution could be calculated as previously mentioned. Both the dose solutions and the dilute dose were kept at 4°C.

10. Scintillation counting solutions:

(a) Organic scintillator: Toluene Phosphor (Panax) Type TTP/4. The solvent is toluene and the solute is:

p-Terphenyl 4 gm.) in 1 litre
1:4 Di-2-(5 phenyloxazolyl) benzene (P.O.P.O.P.) 0.1 gm.)

Toluene phosphor was always kept at 4°C in brown bottles.

(b) Silicone fluid: (Hopkins and Williams) Type MS 200/20 CS. This fluid provided optical contact between jars and photomultiplier of the counter. As dirt affects light transmission of the silicone oil, the photomultiplier was stripped, removed and cleaned with xylene every 6 months and fresh silicone oil pipetted.

Other requirements

Physiological saline. (0.15 M NaCl) sterile for intravenous injection.

Heparin (Waddel Pharmaceuticals) 5,000 units/ml.

Procedure

(a) Administration of the dose

All patients were fasting and resting in bed during the test (9:30-11:30 a.m.). The dose was administered at 9:30 a.m. A sterile 1 ml. pipette was/

was used to transfer 1 ml. of the dose solution to a sterile beaker. The solution was withdrawn into a syringe and the beaker rinsed twice with 2 to 3 ml. of physiological saline and the rinsings withdrawn into the syringe. The dose/test was then administered intravenously over a period of three minutes. When injection was completed, blood was withdrawn into the syringe and reinjected. This was repeated twice to make sure that all the dose was administered.

At half hourly intervals 20 ml. of blood were withdrawn from the patient into a heparinised syringe. The time of withdrawal of the blood samples was always recorded exactly. Four blood samples were taken instead of six 60 ml. samples in the original method (Peterson and Wyngaarden, 1956). Blood was centrifuged immediately at 540 g. for 10 minutes at 20°C. Plasma was separated into 250 ml. glass stoppered flasks.

(b) Extraction of cortisol

100 ml. methylene chloride were added to each plasma sample (about 10 ml.) and extraction carried out by gentle swirling rotation of the flasks for 10 minutes. The contents of the flasks were then transferred to 250 ml. centrifuge bottles, centrifuged at 710 g. for 10 minutes. The contents of the bottles separated into two distinct layers; the plasma constituted the upper thin one, and methylene chloride containing the cortisol formed the lower layer. The upper layer was removed by a fine pipette attached to a water suction pump. The methylene chloride extract was then transferred back to the same flask and washed first with 15 ml. 0.1 N NaOH to remove acidic and phenolic steroids, then with 15 ml. 0.1 N acetic acid and lastly with/

with 15 ml. distilled water. Each washing was always performed in the flasks followed by centrifugation at 710 g. for 5 minutes and the removal of the upper layer by suction. The washed extracts were then transferred from the centrifuge bottles to clean 150 ml. glass stoppered flasks and the extracts evaporated to dryness under reduced pressure by a water pump and in a water bath at 40°C. The dry residues were then redissolved in 3 ml. of methylene chloride, and transferred to 50 ml. glass stoppered test tubes. The flask was rinsed with 2 ml. methylene chloride and the rinsing again transferred to the test tube. The extract was then evaporated to dryness again using the water pump and water bath at 40°C.

10 ml. distilled water were treated in the same way and acted as a blank extract.

(c) Isolation of cortisol by chromatography

Paper chromatography was used for fractionation of plasma steroids and isolation of cortisol. The chromatography system used was the Bush B5 system (Bush, 1952) of benzene:methanol:water (400:200:100); the benzene layer was used as the mobile phase. The chromatography tank used was a rectangular Shandon all glass tank 57 x 30 x 20 cm., containing a longitudinal rounded bottom glass trough about 3 cm. deep, and lined with filter paper dipped into about 1 cm. of benzene. At the bottom of the tank there was a 500 ml. beaker also lined with filter paper and containing 450 ml. of the stationary phase (300 ml. methanol and 150 ml. distilled water) both benzene at the bottom of the tank and stationary phase in the beaker were changed every fortnight. The glass cover of the tank was lined on its lower side by a frame of rubber foam and a plastic sheet and was kept tight by the application of heavy weights on its upper surface to make sure that the atmosphere/

atmosphere inside the tank was kept saturated with the vapours of the solvents. In the middle of the cover there was a stoppered hole, just opposite the middle of the trough, for the introduction of the mobile phase. The chromatography paper used was Whatman No.1 previously treated by exhaustive capillary washing continuously for 3 days with 95% methanol to reduce the blank. The sheet was then cut into smaller sheets each 15.2 cm. wide and 45 cm. long. The starting line was 12 cm. from the upper edge of the paper. The middle section of paper was subdivided into 7 limbs each 2 cm. wide with a 2 mm. strip between each limb (see figure 6). The limbs were cut so that their upper end lay 11 cm. from the upper edge of the paper and their lower end lay 5 cm. from the lower edge of the paper, thus making sure no tangling or cross contamination of the limbs could occur in handling the sheet because their upper and lower ends are joined together.

Support of the chromatography paper during application of the spots was conveniently provided by means of 2 rectangular plywood plates 50 x 30 cm. joined at one end by adhesive plaster so that they could be opened or closed as a book and lined with a sheet of Whatman No.1 chromatography paper. Both the plates and the paper had a window 20 x 3 cm. through which only that part of the paper to be spotted on could show. The edge of the plates containing the window was slightly raised and a metal tube perforated at its upper edge in a slit-like manner was fixed just under the window to direct upwards a draught of the drying current of air coming from a hair dryer placed on its side with its nozzle pointing across the longitudinal axis of the window in the plates. This arrangement kept the chromatography paper clean and allowed/

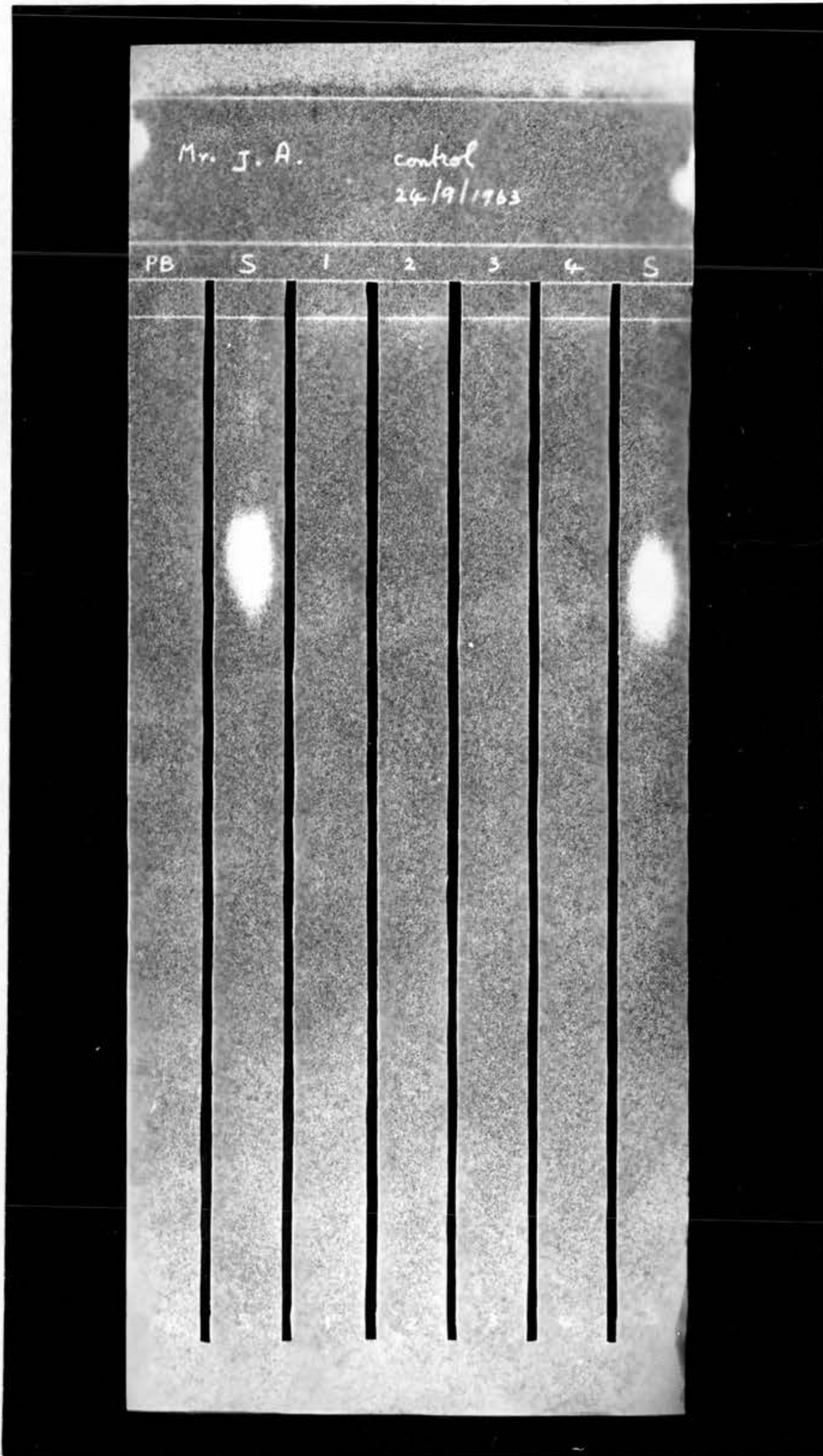


Figure 6 a: Chromatographic separation of plasma cortisol.
Identification by U.V.L. absorption.

allowed drying the spots at the time of their application thus limiting their diameter and keeping their spread to a minimum.

The dry residues were redissolved in 2 x 50 µl. of methylene chloride and transferred to the starting line of a Whatman No.1 chromatography sheet divided into 7 strips; 4 for plasma extracts, one for the paper blank and one at each side for the standard cortisol of which 10 µg. in 10 µl. were used on either limb.

The sheet was then placed in the chromatography tank in such a way that the starting line was at least 6 cm. from the trough edge and the first 4 cm. of the sheet horizontal. Paper was allowed to equilibrate overnight then descending chromatography was started by pouring the solvent (mobile phase) into the trough and chromatography was allowed to proceed in the dark at room temperature for 4 hours. The paper was then taken out of the tank and dried for half an hour in the dark. After drying, the paper was taken to the dark room, pinned over an 8 x 18" sheet of Ilford reflex paper No.50 lying horizontally and photoprinted by exposing it for 25 seconds to ultraviolet light using a lamp emitting nearly all its energy in the 254 mµ region and situated 50 cm. above the paper. The reflex paper was then developed in 200 ml. Copyphen developer (Ilford), diluted 1:5, for 1 minute and fixed as usual. Cortisol standard spots applied to the outer limbs showed as white spots against a dark background (see figure 6 a). The zones containing plasma cortisol and blank on the other limbs lay between the two marker spots. Occasionally when plasma cortisol levels were high, the zones containing plasma cortisol showed also on U.V.L. exposure as faint spots/

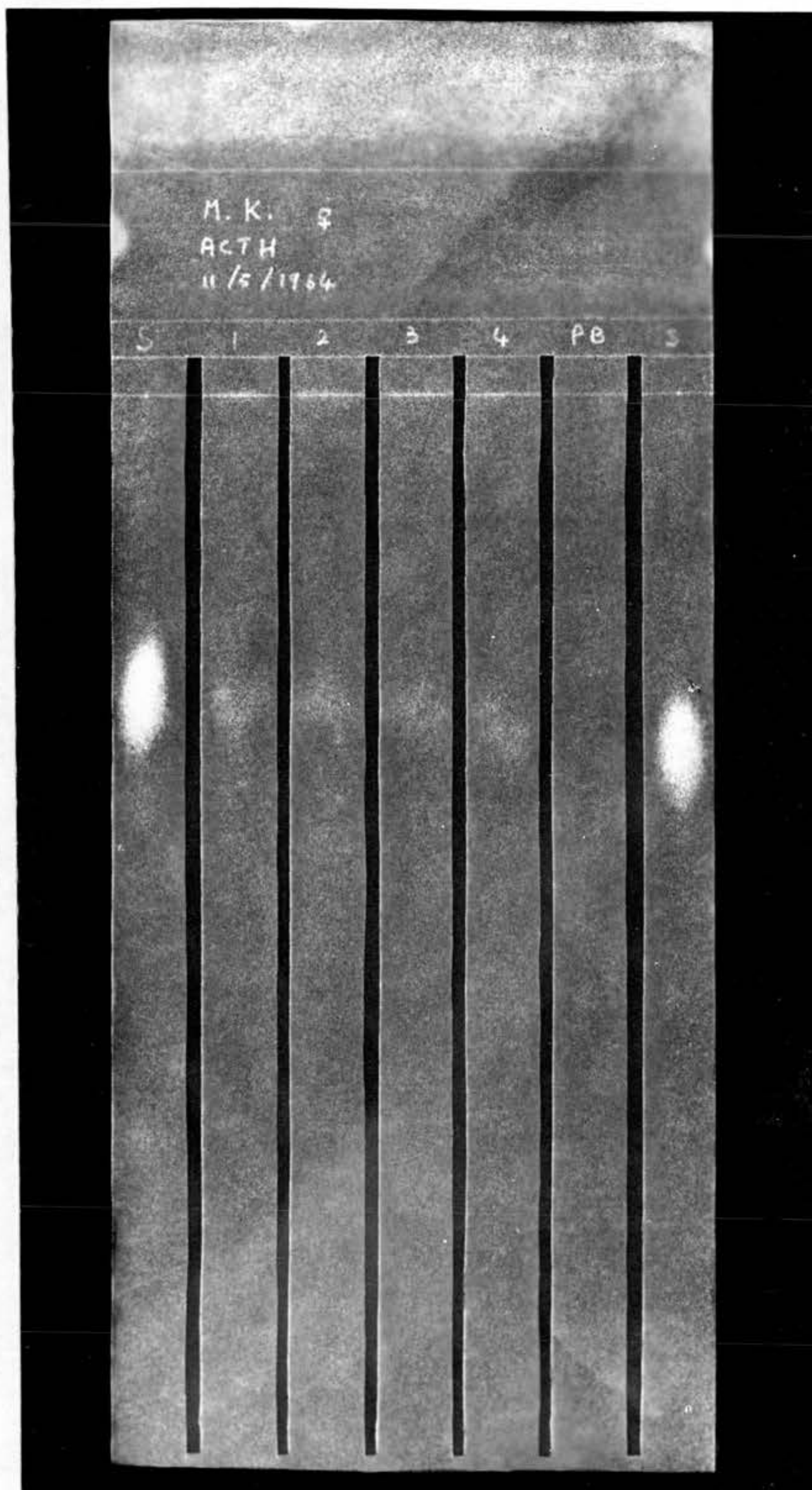


Figure 6 b: Chromatographic separation of plasma cortisol after stimulation with ACTH.

spots especially so with chromatograms of plasma after ACTH stimulation (see figure 6 b). The zone containing plasma cortisol and the paper blank were then cut out, fluted and eluted by immersion in 5 ml. of ethanol overnight.

(d) Measurement of cortisol by spectrophotofluorometry

The theory of fluorescence states that when a molecule or atom is exposed to radiation, an electron transition may occur putting the molecule or atom in a higher energy level (excited state). If the energy which the molecule absorbs is not released by collision with another molecule (or atom) or by other means, then in a fraction of a microsecond, the molecule (or atom) may return to a lower energy state. During the return, radiation of frequency corresponding to the difference in energy of the initial and final energy states is emitted. It is this radiation which constitutes fluorescence (Aminco-Bowman Spectrophotofluorometer, 1956).

The machine used was the Aminco-Bowman Spectrophotofluorometer, consisting of an optical unit, a ballast and control for a Xenon lamp, a photomultiplier microphotometer, a cathode-ray oscillograph and a recording drum. In the optical unit, light from the Xenon lamp is dispersed by the activating monochromator (grating type) into monochromatic radiation incident on the sample. Fluorescent light from the sample is dispersed by a similar monochromator into monochromatic radiation incident on the photomultiplier. The light is transformed there to a weak electrical signal and fed to the photometer where it is amplified. Photometer output is coupled to the cathode ray oscillograph, or is indicated by a self-contained meter/

meter on the photometer in terms of optical densities or percent transmission or recorded by a recording drum.

The efficiency of the fluorometer for estimating accurately small quantities of cortisol was tested by preparing a set of tubes containing 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 μg. of standard cortisol and treating them with ethanol and sulphuric acid, as will be described later, and measuring their fluorescence. The result (see figure 7) shows first that very small amounts of cortisol could be detected and secondly that there is a linear relationship between fluorescence and the concentration of cortisol.

The time needed for the development of maximum fluorescence in the dark was estimated by preparing sets of alcoholic solutions of cortisol standard and keeping them in the dark for varying periods after pipetting sulphuric acid. A curve was drawn which showed that maximum fluorescence occurred after 40 minutes in the dark and was stable for over two hours. Figure 8 shows the relationship between the development of fluorescence and time.

0.2 μg. standard cortisol was used to obtain the activation and fluorescence spectra of 0.2 ml. ethanolic cortisol solution treated with 1.8 ml. sulphuric acid 80%. Figures 9 and 10 show that maximum activation was obtained at a wavelength of 480 mμ. and maximum fluorescence at 540 mμ. using the green filter (OG 1).

Concentration of sulphuric acid needed to produce maximum fluorescence was estimated by preparing several test tubes containing 0.2 μg. standard cortisol in 0.2 ml. ethanol and treating them with 1.8 ml. sulphuric acid of/

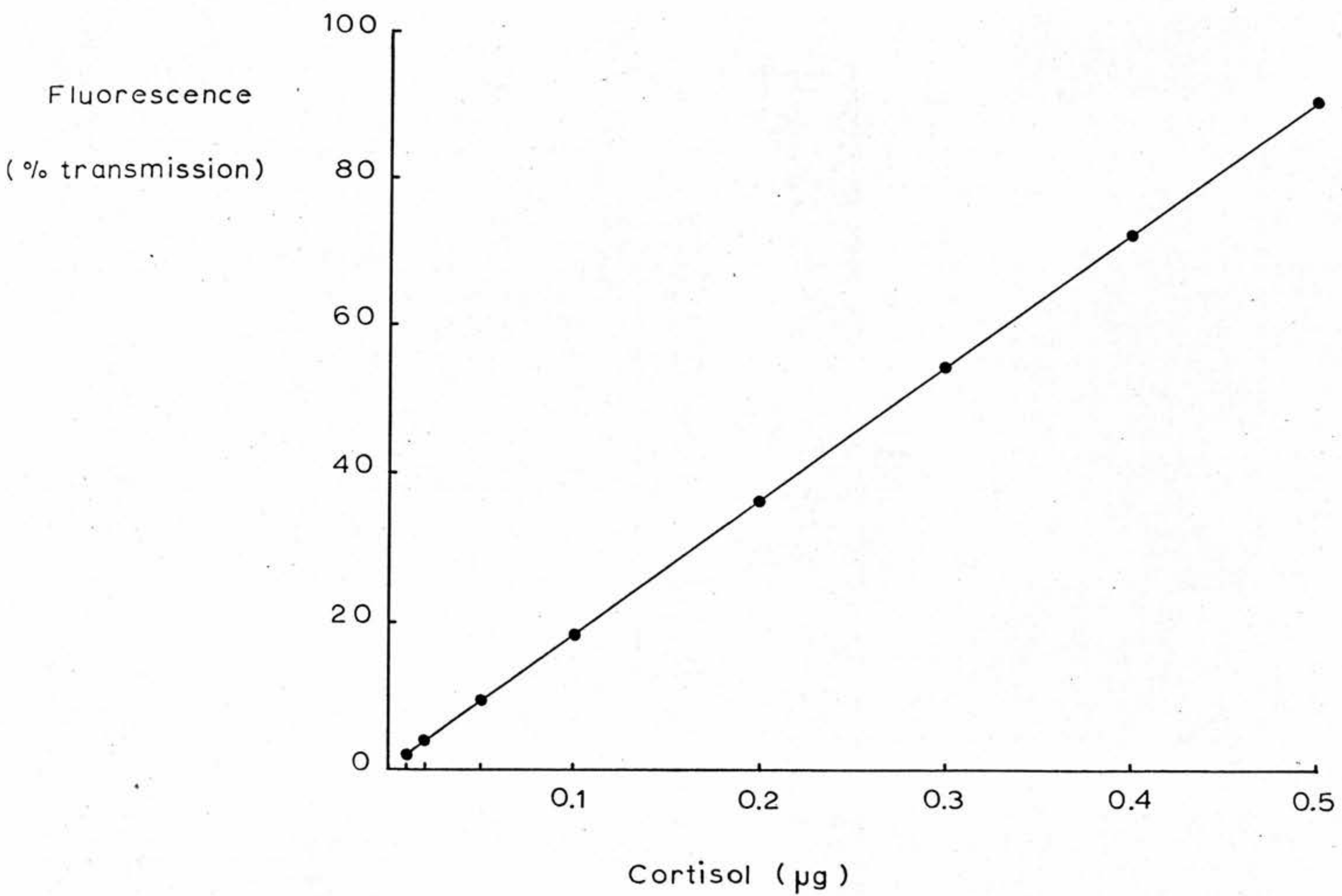


FIGURE 7: FLUORESCENCE OF DIFFERENT CONCENTRATIONS OF CORTISOL

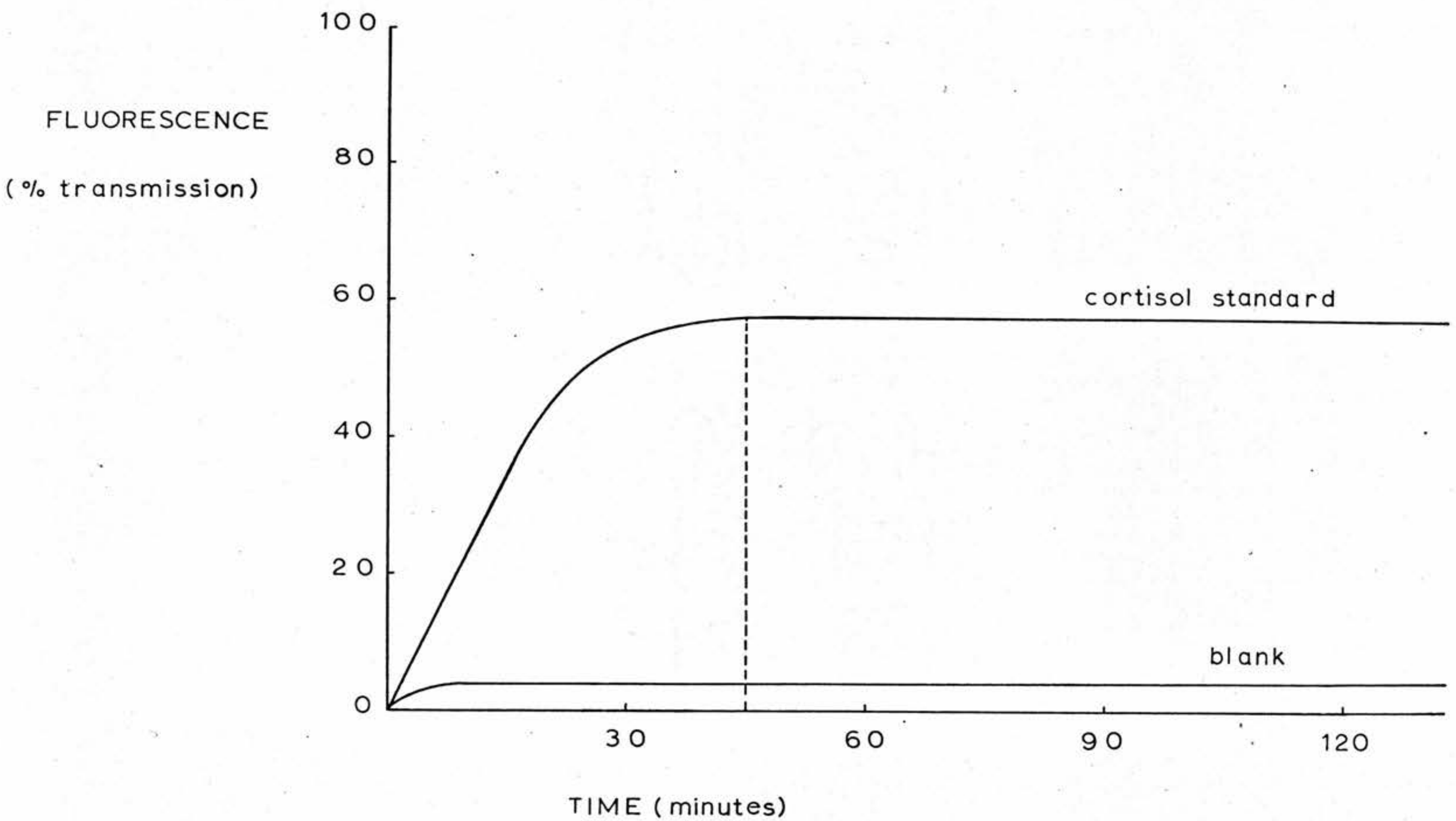


FIGURE 8: TIME OF DEVELOPMENT OF FLUORESCENCE

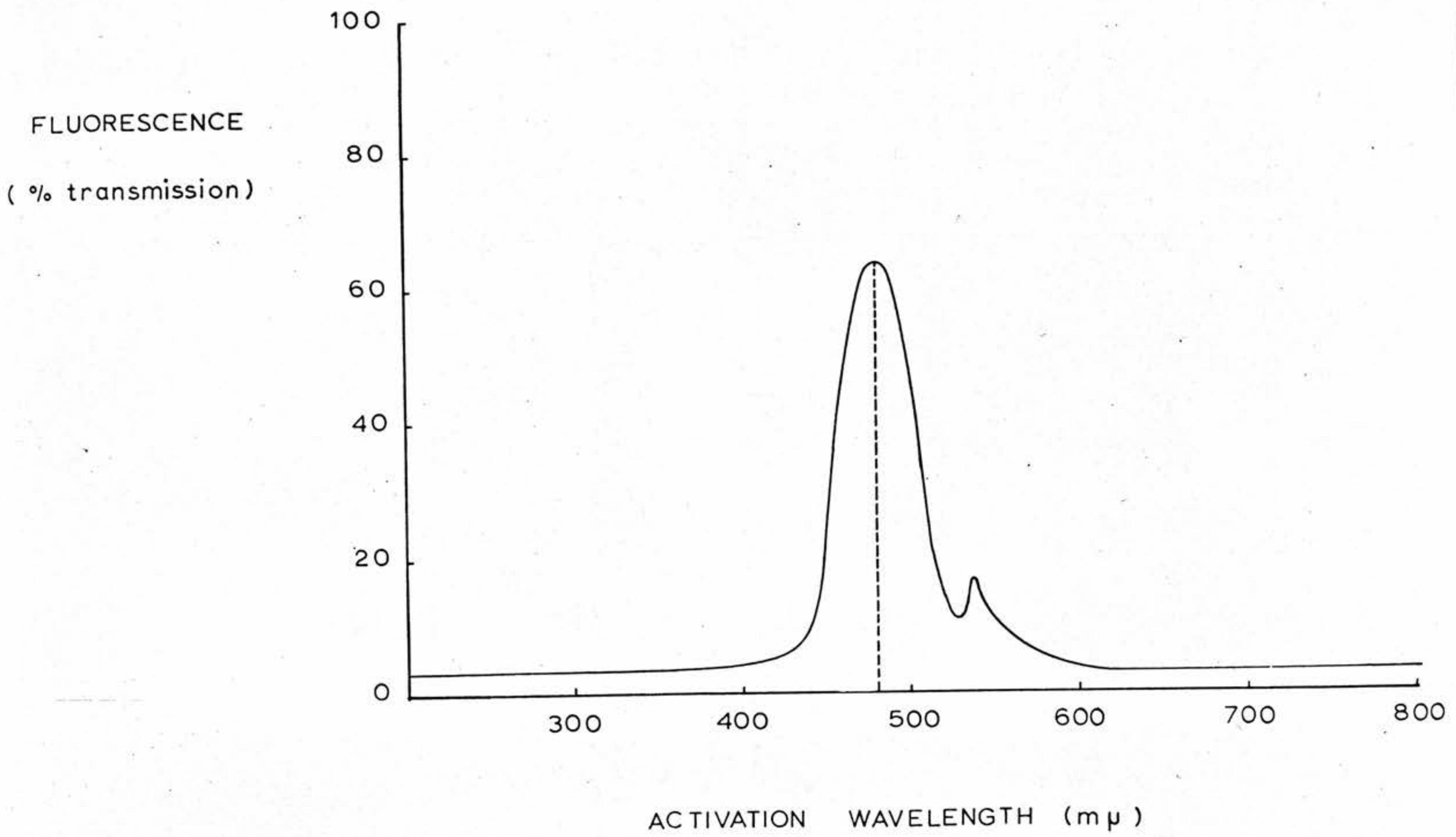


FIGURE 9 : ACTIVATION SPECTRUM OF CORTISOL
(at fluorescence wavelength 540 mμ)

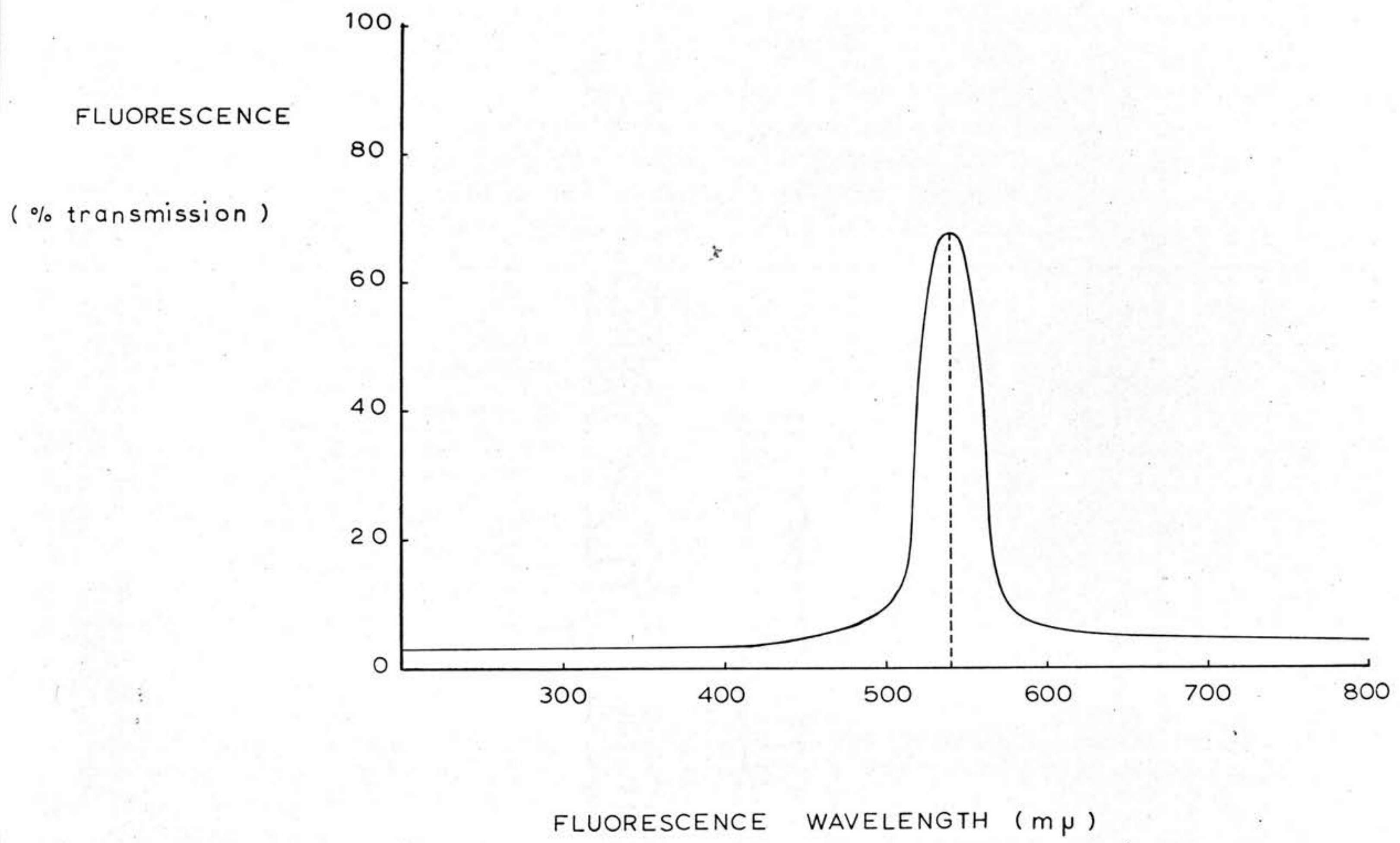


FIGURE 10 : FLUORESCENCE SPECTRUM OF CORTISOL

(at activation wavelength 480 mμ)

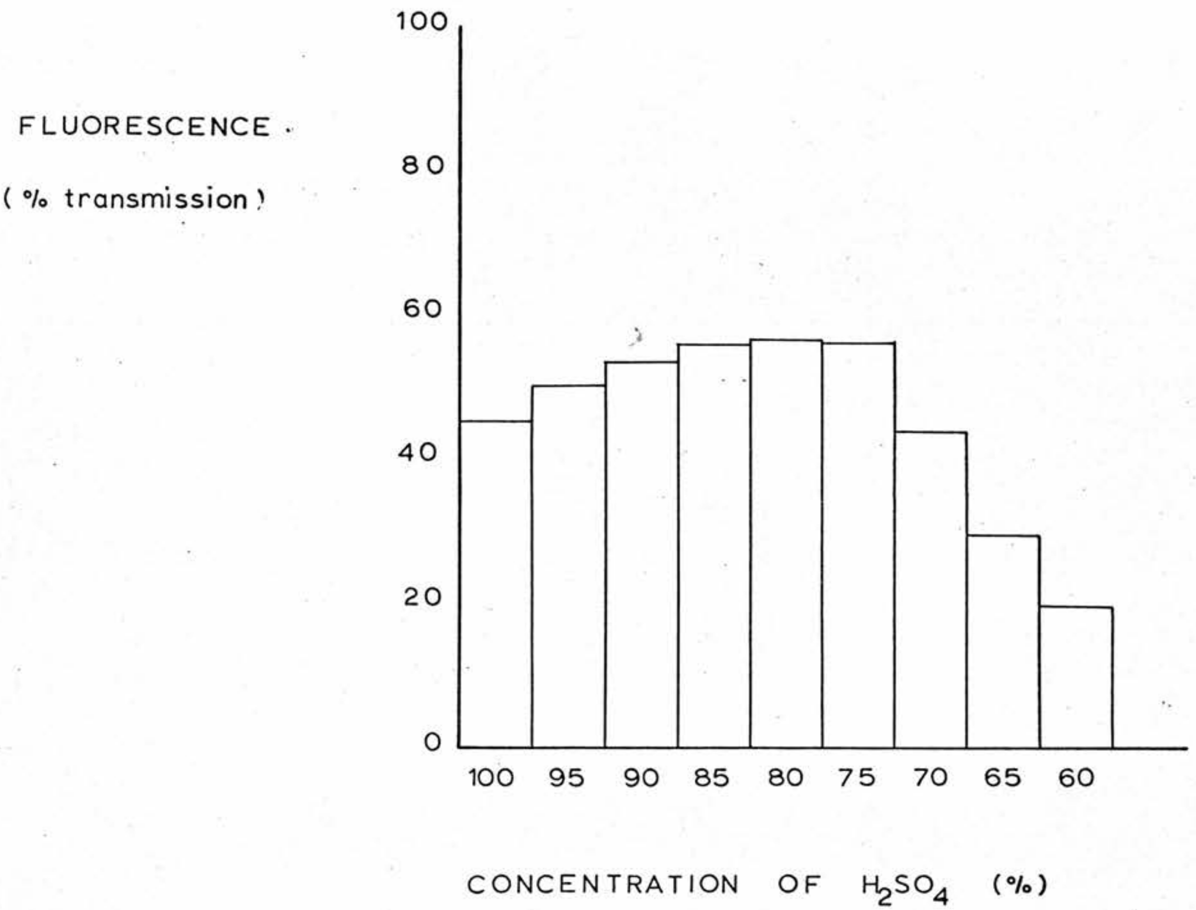


FIGURE 11: EFFECT OF CONCENTRATION OF H_2SO_4 ON FLUORESCENCE OF CORTISOL

of gradually increasing concentration. Figure 11 shows that maximum fluorescence was obtained with 3 concentrations of sulphuric acid 75, 80 and 85%. Therefore sulphuric acid of a concentration of 80% was used throughout this study.

For fluorometry, eight 50 ml. glass stoppered test tubes were used, four for the plasma samples, one each for paper blank, standard (≡ 0.2 µg.), blank (ethanol) and dilute dose solution; into each 1 ml. of the corresponding eluate or solution was pipetted. 0.5 ml. of eluate of plasma and of paper blank were used in estimations after ACTH stimulation. The contents of these test tubes were evaporated to dryness under reduced pressure on a water bath at $40^{\circ}C$. 0.2 ml. ethanol was added to each and tubes rotated thoroughly to dissolve the residues. Then 1.8 ml. 80% H_2SO_4 were pipetted rapidly and contents mixed well. Tubes were then left in the dark for 45 minutes at room temperature to allow fluorescence to develop. At the end of this time contents of the tubes were transferred to the quartz cells of the fluorometer (cells previously washed with tap water, distilled water, twice with ethanol and dried). The fluorescence of each was measured at an activation wave-length of 480 mµ. and fluorescence wave-length of 540 mµ. using the green filter (OG 1). The value of the blank was subtracted from that of the standard and dilute dose solution, and that of the paper blank subtracted from each of the plasma samples.

Cortisol content of the plasma samples in µg./1 ml. eluate equals

$$\frac{0.2 \times (\text{fluorescence of eluate} - \text{fluorescence of paper blank})}{(\text{fluorescence of standard} - \text{fluorescence of blank})}$$

which was then multiplied by three to get the amount per 3 ml. eluate.

(e)/

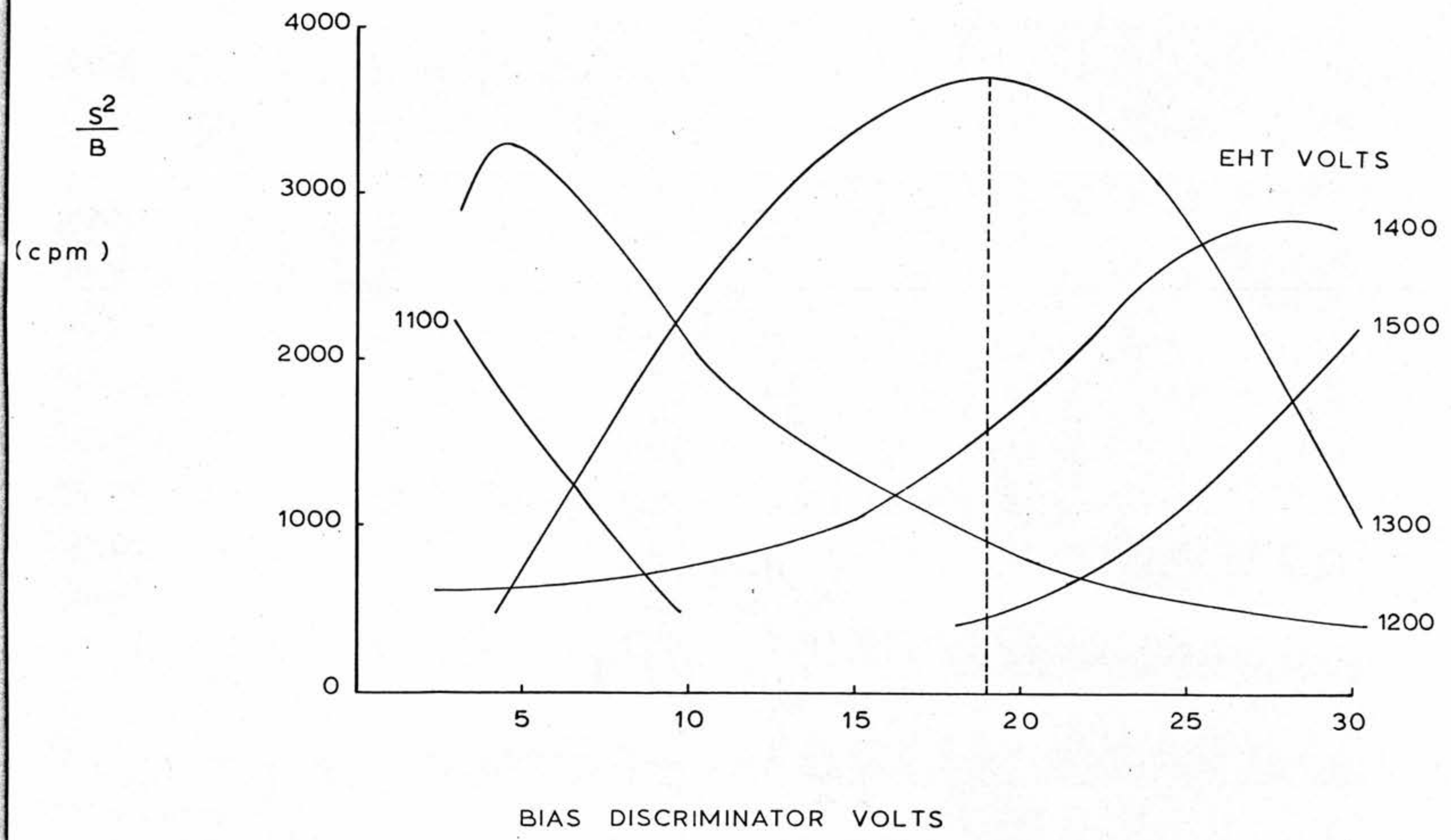


FIGURE 12 : OPTIMUM EHT AND BIAS DISCRIMINATOR VOLTS

(e) Radioactivity counting

The machine used, was the assembly Type SC/LP, Panax Equipment Ltd., consisting of:

1. An automatic counter, type AC 300/6, which comprises an automatic timer, type T 300, and a decatron scaler type D 657.
2. A lead castle and an amplifier, type 4250, with a gain of x 1000.

For nuclides such as C^{14} (0.155 MeV) the basic requirements are a head amplifier, a scaler and a high stability power unit.

Tap water was allowed to pass continuously, for 1 hour before and during counting, through the cooling coils of the head unit of the castle. Activity was counted in glass stoppered 15 ml. Panax jars. Optical contact at the window between the jar and the photomultiplier tube was provided by the silicone oil.

Optimum working conditions for counting of C^{14} by the machine had to be determined and checked once every 6 months. This was carried out by counting a background and a source of C^{14} at varying E.H.T. volts (extra-high tension volts) and varying Discriminator Bias volts and recording

$$\frac{(\text{cpm of the } C^{14} \text{ source} - \text{cpm of the background})^2}{\text{cpm of the background}} \quad (\text{i.e. } \frac{S^2}{B}) \text{ for each E.H.T.}$$

against the Discriminator Bias volts. An example is given in figure 12 which shows the different curves obtained. From this it is seen that conditions needed for optimum working are provided by an E.H.T. = 1300 volts and Disc.Bias = 19 volts.

Counting C^{14} was performed by the liquid scintillation method. This method is based on the small flashes of light (or scintillations) which accompany/

accompany the exposure of certain materials to nuclear radiation. The mechanism of formation of these scintillations is complex, but essentially it involves the absorption of most of the energy from the nuclear radiation by the solvent of the phosphor. This energy is then transferred to the solute leading to the formation of a high-energy (or excited) electronic state of its molecules (or atoms). The excess energy is then emitted, within a very short time, as a flash of light. It is due to the use of the electron multiplier tube with a photoelectric cathode (photomultiplier tube) that light produced in a single scintillation, which is too feeble to be measured directly, causes the emission of electrons from the cathode of the tube due to the photoelectric effect. The number is greatly increased in successive steps, so that ultimately a measureable current pulse is produced which can be recorded by means of a suitable counter (Glasstone, 1958).

Toluene phosphor was used as the organic scintillator; 5 ml. of which were used, blended with either 4 ml. of ethanol for the background counting or with smaller volumes of active samples (dissolved in ethanol) and completed to 4 ml. with ethanol.

3 ml. of the eluates were used for counting. 6 counting jars were used 4 for the plasma samples, one for the diluted dose solution and one for the background. They were prepared as follows:-

	alcohol ml.	sample ml.	Toluene phosphor ml.
Background	4	-	5
Sample	1	3	5
Diluted dose solution	3	1	5

Contents/

Contents of the jars were mixed by gentle swirling till the toluene phosphor and ethanol were completely blended. Jars were then placed in the dark for 10 minutes (in order to reduce fluorescence in the phosphor) in a petri dish containing tap water to bring them to the same temperature as that in the castle. Jars were then housed in the castle and further 10 minutes were allowed to pass for dark adaptation before counting started. Radioactivity was measured initially in terms of the time required to register a certain number of counts as follows: the background - 4000 counts, plasma samples - 10,000 counts and dilute dose solution - 40,000 counts. The value of background (cpm) was always subtracted from the values of the other active substances. The activity of the plasma was expressed as cpm/3 ml. eluate.

Counting of the background was almost always 100 cpm (ranged between 96 and 104), those of the plasma eluates ranged from 240 - 52 cpm for the first sample, to 67 - 11 cpm for the last one using 1 μc. as a dose and 321 - 63 cpm for the first sample, to 52-18 cpm for the last one using 2 μc. Dilute dose solution was around 7000 cpm. The validity of the count rate of the plasma samples, especially the last one, looked questionable in relation to the count rate of background. Thus, an experiment was performed in which 4 ml. of a standard solution containing fairly high amount of radioactivity was counted, together of course with a background. Steadily decreasing volumes of the same standard i.e. 3 ml., 2 ml., 1 ml., 0.5 ml., 0.2 ml., and 0.1 ml. were also counted and the value of background subtracted from each. The observed count rates were compared with the theoretical/

theoretical ones based on the count rate of 4 ml. standard. The results, shown on table 6 indicate a fairly high efficiency for counting small count rates down to 6 cpm.

Table (6)

Efficiency of counting solutions of low activity

Radioactive standard solution (ml.)	(cpm)	Observed activity (cpm)	Efficiency (%)
4	240	240	100
3	180	186	103.3
2	120	118	98.2
1	60	62	103.3
0.5	30	30	100
0.2	12	13	108.1
0.1	6	6	100

98.2 - 108.1

(f) Estimation of the specific activity of cortisol

The specific activity of each plasma sample was calculated by dividing the total count of the sample by the corresponding fluorometric estimation. Thus:

$$\text{specific activity (S.A.) eluate} = \frac{\text{Activity (cpm/3 ml.) eluate}}{\text{Cortisol } (\mu\text{g./3ml.})\text{eluate}} \quad \text{cpm}/\mu\text{g}$$

and for the dose solution:

$$\text{S.A. dose} = \frac{\text{Activity (cpm/1 ml.) dilute dose solution}}{\text{Cortisol } (\mu\text{g./1 ml.}) \text{ dilute dose solution}} \quad \text{cpm}/\mu\text{g.}$$

(g) Calculation of the body miscible pool, rate of turnover, biological half clearance time and production rate of cortisol

These were calculated using the isotope dilution expressions:

(1)/

(1) $A = a \left[\frac{Ii}{Io} - 1\right] \times 10^{-3}$ mg.

for estimation of the miscible pool, and

(2) $k = \frac{1000}{t\frac{1}{2}}$ pool/24 hrs.

for estimation of the rate of turnover.

Cortisol production rate = (1) x (2) mg./24 hrs. (see Chapter II of the introduction).

Example:

Suppose that:

The amount of cortisol in the dose (a) is 30 μg.

The specific activity of the dose (Ii) is 20,200 cpm/μg.

The specific activity at zero time (Io) is 200 cpm/μg.

Biological half clearance time ($t\frac{1}{2}$) is 100 minutes

then the miscible pool of cortisol will be:

$$A = 30 \left[\frac{20,200}{200} - 1\right] \times 10^{-3}$$

= 3 mg.. (1)

and the rate of turnover will be:

$k = \frac{1000}{100}$ = 10 pool/24 hrs.............................. (2)

and the production rate will be (1) x (2), i.e.

3 x 10 = 30 mg./24 hrs.

Radioactive waste and protection:

Besides the fact that C^{14} is a very weak β-emitter (0.155 MeV), the amounts handled were very small. Accordingly, and after consultation with the Medical Physics Department of the Royal Infirmary of Edinburgh, it was agreed/

agreed that it is quite safe from the public health point of view, both to store the various solutions containing radioactive cortisol in the refrigerator used for storing various chemicals and to wash the radioactive waste down the sink using plenty of water. So, glassware which contained radioactive C^{14} were thoroughly cleaned by brushing using teepol, and plenty of tap-water then rinsed with distilled water. Pipettes were treated in the same way, then rinsed at the end with acetone.

Film badges, supplied by the Physics Department of the Western General Hospital of Edinburgh, were used by all people handling C^{14} and were changed every fortnight and checked for the degree of exposure to radioactivity.

ii. Estimation of cortisol production rate by the urine method

The method applied here is based largely on that described by Cope and Black (1958 a).

Principle

The principle of the urine method is the same as that of the blood method except that the degree of dilution of the administered dose refers to cortisol metabolites (particularly tetrahydrocortisone which is the main metabolite of cortisol) instead of to cortisol as in the blood method (see Introduction, Chapter II).

After administration of the dose, a 24 hour urine sample was collected. The total radioactivity was measured and conjugates of cortisol metabolites in urine were hydrolysed using β-glucuronidase, ~~cortisol metabolites~~ extracted and separated by chromatography. The S.A. of tetrahydrocortisone (THE) was then determined by estimation of both its radioactivity and its content spectrophotometrically. Cortisol production rate was calculated as the total radioactivity in the 24 hour sample divided by the S.A. of THE, i.e.

$$\text{Cortisol production rate} = \frac{\text{Total activity of 24 hour urine (cpm)}}{\text{S.A. THE (cpm/}\mu\text{g.)}} \times 10^{-3} \text{ mg./24 hrs.}$$

Reagents

1. Tetrahydrocortisone standard

    (a) Stock standard: 0.4 mg. THE (Steraloid)/ml. absolute ethanol.

    (b) Working standard: 20 µg. THE/ml. absolute ethanol.

2. Tetrahydrocortisone marker: 1 mg. THE/ml. absolute ethanol.

THE standards and THE marker were kept always at 4°C.

3. β-glucuronidase: (Limpet), 2,000,000 units/g. (Baylove Chemicals)

4./

4. Chloroform, R (M & B)

5. Sodium hydroxide (B.D.H.) 0.1 N approximately.

6. Ethane-diol (Ethylene glycol) (B.D.H.).

7. Toluene (B.D.H.) Analar grade.

8. Methanol (B.D.H.) Analar grade.

9. Glacial acetic acid (M & B)

10. Purified alcohol.

11. Tetramethyl-ammonium hydroxide (B.D.H.) 25%

12. Blue tetrazolium [3:3'-Dianisole-Bis-4:4'- (3:5 Diphenyl) - Tetrazolium Chloride] (B.D.H.)

13. 10% Sodium hydroxide in 60% methyl alcohol (1:1 v/v)

14. Methanol/ethylene glycol (1:1 v/v)

15. Acetate buffer : pH 4.6; 0.5 M

16. Hydrochloric acid: 0.1 N approximately.

17. Radioactive compounds

    a. Dose solution) Prepared as described before
    b. Dilute dose solution)

    c. Internal Standard: with doses of 1 μc., the dose solution was diluted with absolute ethanol to give about 650 cpm/ml. With doses of 2 μc. it was diluted to give about 1800 cpm/ml.

18. Scintillation counting fluids: as in the blood method.

Procedure

(a) Administration of the dose and collection of urine

Patients were made to empty their bladders and dose administered as in blood method. Urine was collected without preservative for 24 hours and total volume noted.

(b)/

(b) Estimation of total radioactivity

10 ml. of the urine were filtered and the total radioactivity determined on 0.5 ml. of filtrate. Urine does not mix with toluene, but by blending 5 ml. of the phosphor with 4 ml. ethanol, up to 0.5 ml. urine may be incorporated by gentle swirling to ensure good mixing. The incorporation of 0.5 ml. urine into ethanol and liquid phosphor, however, produced some turbidity, presumably because of the presence of dissolved salt and other substances in urine and this sometimes exerts a considerable quenching effect.

Correction for this quenching was made by adding an internal standard. Counting the urine was carried out before and after the addition of the standard, the observed count of the standard in urine was compared with its true value and so a correction factor for quenching was obtained. Accordingly to obtain the total radioactivity in urine, 5 jars were prepared as follows:-

	alcohol ml.	urine ml.	Int.std. ml.	Dilute dose ml.	Toluene Phosphor ml.
1. Background (BG)	4	-	-	-	5
2. Urine (U)	4	0.5	-	-	5
3. Internal standard (S)	3	-	1	-	5
4. Urine + standard (U + S)	3	0.5	1	-	5
5. Dilute dose (D)	3	-	-	1	5

The value of the background (cpm) was subtracted from the radioactivity (cpm) of the other samples.

Quenched/

Quenched standard = cpm (U + S) - cpm (U)

Urine count corrected for quenching was $\frac{\text{cpm (U) x cpm (S)}}{\text{cpm of quenched (S)}}$

As only 0.5 ml. of urine was used for counting, the total radioactivity of sample (expressed as cpm/24 hrs.) was:

Total count of 0.5 ml. urine x 2 x total volume of 24 hour sample.

The background was counted till 4,000 counts were recorded, urine samples and standard till 10,000 and dilute dose solution till 40,000. Jars were kept in the dark in a petri dish containing tap water for 10 minutes, then housed in the castle for another 10 minutes before counting started. Counting of the background was almost always 100 cpm (ranged between 96 and 104), that of the urine sample varied according to the volume of urine and percent of dose excreted in 24 hours, but generally more than 300 cpm were recorded with 1 μc. dose and more than 700 cpm were recorded using 2 μc. dose. The internal standard was prepared such that the counting of urine + internal standard should always be more than double the counting of urine. By this, the maximum correction for quenching could be made. Thus an internal standard of 900 cpm/ml. and 1800 cpm/ml. were found to be suitable to use with the different activities of the urine samples estimated after administration of doses of 1 μc. and 2 μc. respectively.

The validity of this method for correction of the quenching was tested by preparing 8 sets of jars each is composed of:

1. Background.
2. 0.5 ml. different urine sample plus a quantity of radioactivity equivalent to 900 cpm.
3. 0.5 ml. urine, plus a quantity of radioactivity equivalent to 900 cpm plus 1 ml. internal standard (1800 cpm/ml.)

4./

4. 1 ml. internal standard.

Ethanol was added to 4 ml., and then 5 ml. toluene phosphor were added and jars counted as usual. Countings of the added radioactivity were recorded before and after correction for quenching using the internal standard and the results compared to the original activity added (900 cpm). Results are shown in table 7.

Table (7)

Validity of the correction method for quenching of urine

Urine sample (No.)	Added radioactivity (cpm)	Observed radioactivity (cpm)	Corrected radioactivity (cpm)	Efficiency (%)
1	900	846	917	101.8
2	900	839	905	100.5
3	900	869	883	98.1
4	900	851	894	99.2
5	900	815	926	102.8
6	900	842	919	102.4
7	900	836	928	103.3
8	900	853	897	99.6
				98.1 - 103.2

These results clearly indicate that the employment of an internal standard to correct the quenching is essential and also that this method of correction is reliable.

(c) Enzymatic hydrolysis

For estimations at basal conditions, one tenth of the 24 hour urine sample was used. Its pH was adjusted to 4.6 with 0.1 N HCl (using narrow-range pH papers; B.D.H., 4 - 5.5) and the pH stabilised by the addition of an equal volume of acetate buffer (pH 4.6, 0.5 M). β-glucuronidase (500 units to each ml. of urine, dissolved in few mls. acetate buffer) was then added and thoroughly/

thoroughly mixed with the urine, which was then incubated at 37°C for 24 hours.

After ACTH stimulation, urine contained increased amounts of cortisol metabolites, so that one twentieth of the total 24 hour urine sample was quite adequate for this estimation. On the other hand markedly diminished quantities of cortisol metabolites in urine after dexamethasone suppression justified the use of one fifth of the total 24 hour urine sample for this test.

(d) Extraction of cortisol metabolites

This was carried out in one-litre separating funnels using 2 x 50 ml. chloroform. The lower chloroform layer was transferred to centrifuge bottles and the emulsion formed during extraction broken by centrifugation at 710 g. for 15 minutes. The upper layer was removed by suction and the lower chloroform layer washed in a 250 ml. glass stoppered flask twice with 15 ml. of 0.1 N NaOH, to remove acidic and phenolic steroids, and twice with 15 ml. distilled water. Centrifugation was carried out after each washing and the upper layer was removed by suction. After the last washing, the extract was transferred to a clean 150 ml. glass stoppered flask and brought to dryness under reduced pressure in a water bath at 50°C.

An equal volume of distilled water was treated as urine and served as the blank.

(e) Isolation of tetrahydrocortisone by chromatography

Paper chromatography was used for the fractionation of the urinary metabolites of cortisol. Isolation of tetrahydrocortisone, the main metabolite of cortisol, was the main factor which determined the solvent system to be used, namely that of Zaffaroni (Burton et al, 1951 a) subsequently modified/

modified by Nowaczynski and Koiw (1957) in which ethylene glycol/toluene as the partition system replaced Zaffaroni's original propylene glycol/toluene. This modification shortened the running time from 96 hours to 13 hours without loss of sharpness of separation.

Paper used was Whatman No. 54; 45 x 15 cm. previously washed for 3 days with 95% methanol while hanging in a chromatography tank. The paper was divided into 4 limbs with spaces of 1 cm. between each. The outer two limbs were 2 cm. wide and were used for spotting tetrahydrocortisone marker and the inner two were 4 cm. wide, one ~~of them~~ for spotting the sample and the other for the paper blank. The starting line was 12 cm. from the upper edge of the paper and the limbs started 11 cm. from the upper edge of the paper and ended 5 cm. from its lower edge (see figure 13). Paper had first to be impregnated with the stationary phase (methanol/ethylene glycol 1:1 v/v) and the wet paper pressed between two sheets of previously washed Whatman paper to remove excess solution.

Dried chloroform extracts were then redissolved in 2 x 0.2 ml. chloroform in acetone(99:1 v/v)and transferred to the starting line of the chromatography paper. 10 µl. tetrahydrocortisone marker was applied to the starting line of each outer limb and paper was then transferred to the chromatography tank, which is similar to that used with the Bush B5 system. No beaker was used at the bottom of the tank and filter paper lining the tank dipped into about 1 cm. of mobile phase, toluene saturated with ethylene glycol. As equilibration is not needed with this solvent system, descending chromatography was started by pouring the mobile phase, toluene saturated with ethylene glycol, through the stoppered hole in the middle of the/

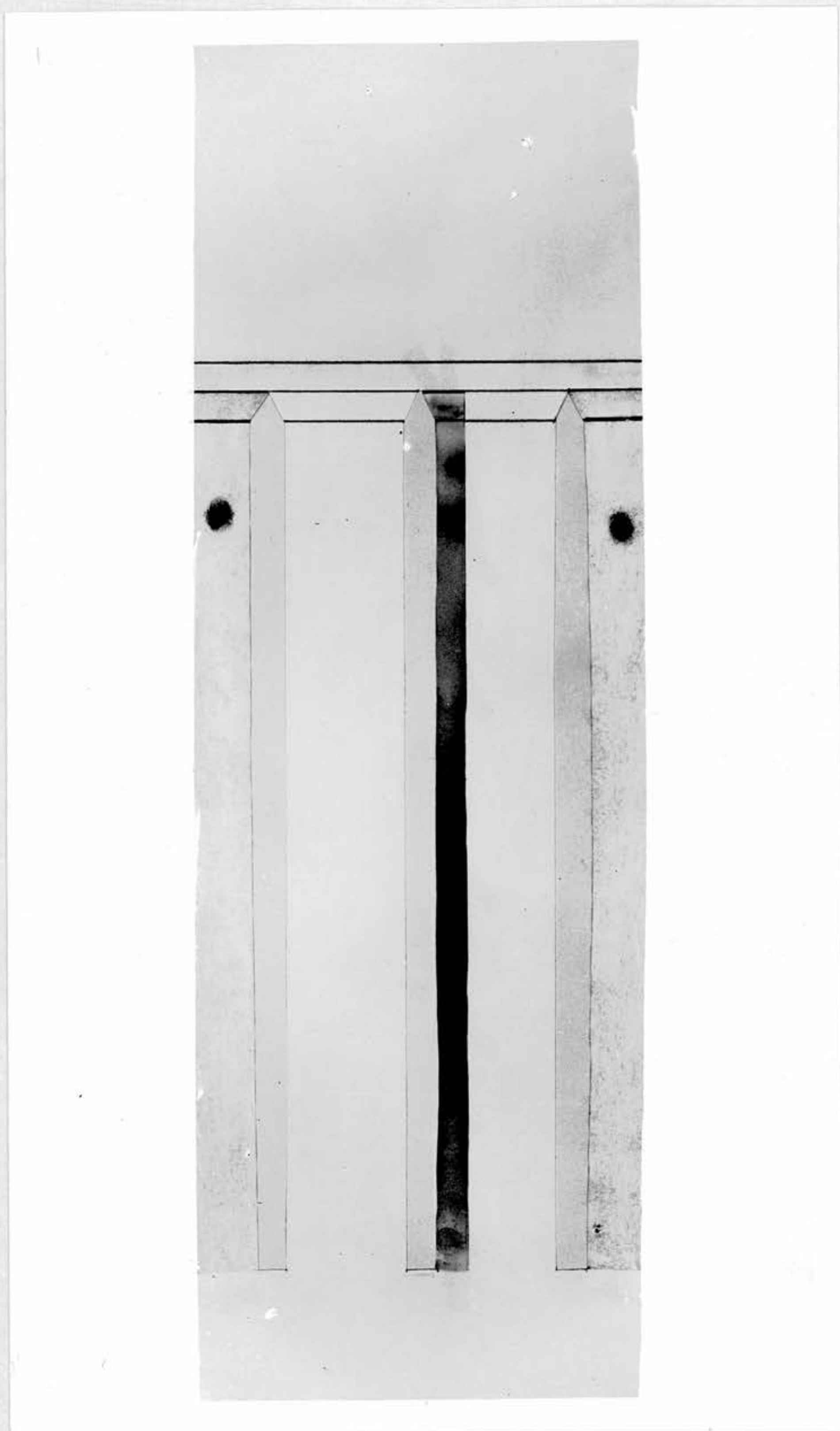


Figure 13: Chromatographic separation of urinary THE. Identification by the BTZ reaction.

the cover directly to the trough and chromatography was allowed to proceed in the dark at room temperature for 13 hours. Paper was then taken out of the tank and dried in the dark. But owing to the presence of ethylene glycol drying took at least 6 hours using a hair dryer.

To locate the tetrahydrocortisone zone, a 1 cm.-wide strip of the urine sample limb was cut off and dipped, together with the outer 2 standard tetrahydrocortisone marker limbs in a solution consisting of 15 mg. blue tetrazolium dissolved in 3 ml. distilled water to which 9 ml. of 10% sodium hydroxide in 60% aqueous methanol were added. In few seconds, in the strip of the urine sample limb, 3 purplish-mauve bands appeared; the one nearest to the origin was a dense narrow one due to the water insoluble formazan formed by the interaction of tetrahydrocortisol with the blue tetrazolium. The middle one was broader and much denser and was due to the formazan formed by the interaction of blue tetrazolium with tetrahydrocortisone. A third faint band further away from the origin was occasionally seen and was due to allo-tetrahydrocortisol. Similarly, THE standard marker applied to the outer two limbs appeared as purplish-mauve spots at the same horizontal level as the THE of the urine chromatogram (see figure 13). The bands of the urine chromatograms were, on the whole, much denser for urines after ACTH stimulation and very faint after dexamethasone suppression. The section of the urine sample limb and of the paper blank, opposite the developed tetrahydrocortisone band and standard marker spots, were cut out, fluted and eluted by immersion in 5 ml. ethanol overnight.

(f)/

(f) Estimation of the radioactivity of the eluate

For this purpose 3 ml. of the eluate were taken for counting; 2 jars were set as follows:

	alcohol ml.	eluate ml.	toluene phosphor ml.
Background	4	-	5
Eluate	1	3	5

and counting carried out as previously described.

(g) Estimation of THE

0.5 ml. of the eluate was taken for spectrophotometric estimation of tetrahydrocortisone relative to THE standard. The procedure used in this study for the quantitative estimation of THE was a modification of that used by Nowaczynski et al (1955). In principle it is based on the fact that alcoholic solutions of certain steroids reduce tetrazolium salts in the presence of tetramethyl-ammonium hydroxide forming coloured formazans. 4 tubes were prepared as follows:-

Tube	alcohol ml.	eluate ml.	standard* ml.	BTZ** ml.	TMAH*** ml.
Blank	2.5	-	-	0.25	0.25
Paper blank	2	0.5	-	0.25	0.25
Standard	1.5	-	1.0	0.25	0.25
Eluate	2	0.5	-	0.25	0.25

* Standard THE solution used was 20 µg./ml.

** Blue tetrazolium solution was prepared immediately before use by dissolving 50 mg. blue tetrazolium in 10 ml. ethanol.

***Tetramethyl-ammonium hydroxide solution was prepared immediately before use by using 0.4 ml. of the 25% solution and completing the volume to 10 ml. with ethanol.

This/

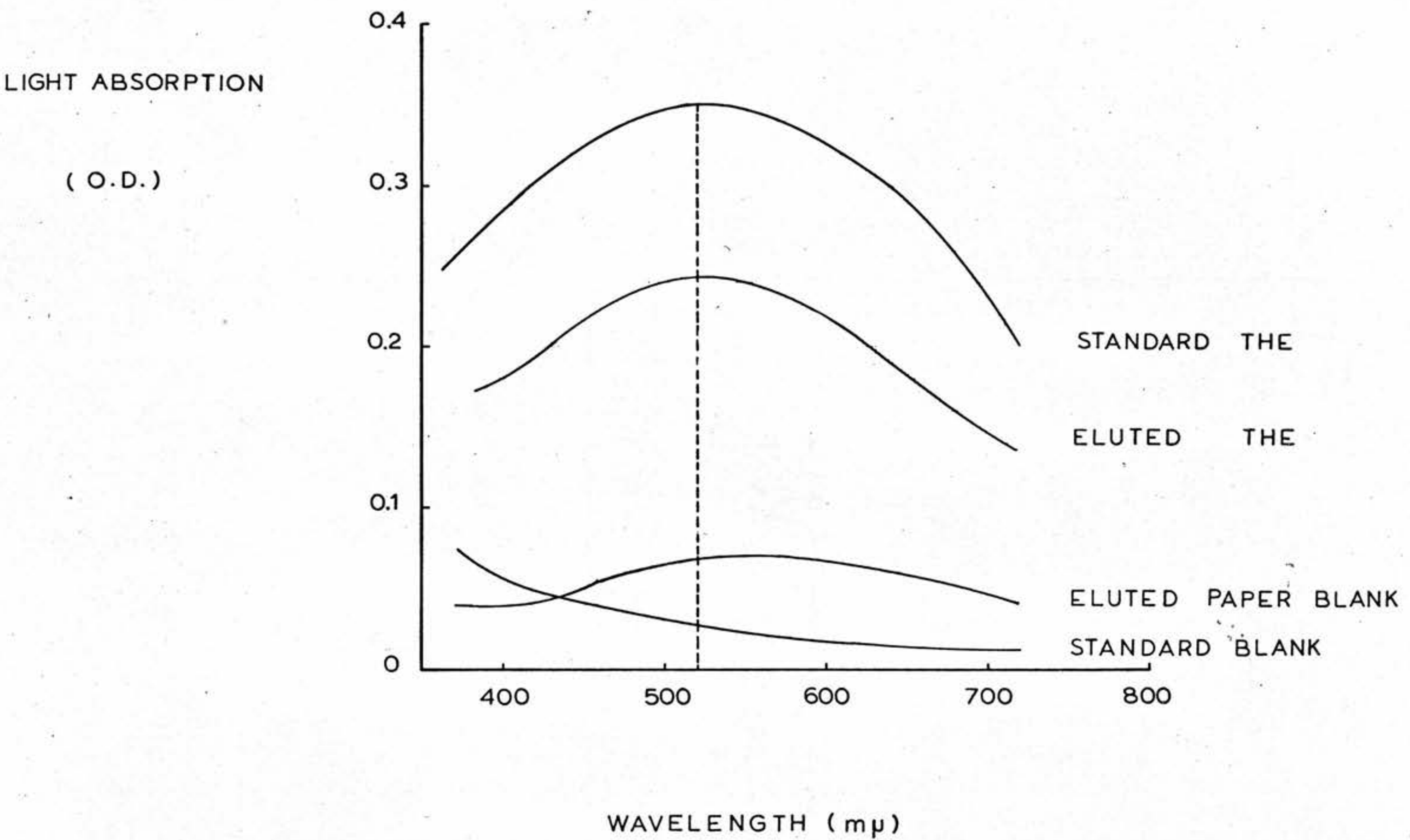


FIGURE 14: ABSORPTION SPECTRUM OF THE REACTION WITH BLUE TETRAZOLIUM

This arrangement was convenient to use for estimation at basal conditions and after ACTH stimulation. With urines containing small amounts of THE as a result of dexamethasone administration, 1 ml. of eluate was used instead of 0.5 and ethanol added was 1.5 ml. Solutions were thoroughly mixed and kept in the dark for 25 minutes, at the end of which the reaction was stopped by pipetting 1 ml. glacial acetic acid into each tube. Optical densities were read against an ethanol blank in an S.P. 600.

$$\text{Amount of THE in 0.5 ml. eluate} = \frac{20 \times (\text{O.D. of sample} - \text{O.D. of paper blank})}{(\text{O.D. of standard} - \text{O.D. of blank})} \ \mu g.$$

which if multiplied by 6 gives the amount per 3 ml. eluate.

(h) Estimation of specific activity of THE

Specific activity of THE could be calculated as follows:

$$\text{S.A. THE} = \frac{\text{Radioactivity in 3 ml. eluate (cpm)}}{\text{THE in 3 ml. eluate } (\mu g.)} \quad (\text{cpm}/\mu g.)$$

The spectrophotometer used was the Unicam S.P. 600, designed for absorptiometric measurements within the visible and near-infra-red regions of the spectrum. A wave-length of 520 mμ. and the blue photocell were used.

Figure 14 shows a plot of optical densities against wave-lengths using the S.P. 600. An absorption maximum at 520 mμ. for eluted and standard tetrahydrocortisone was obtained.

(i) Calculation of cortisol production rate

$$\text{CPR} = \frac{\text{Total count in 24 hour urine (cpm)}}{\text{S.A. THE } (\text{cpm}/\mu g.) \times 1000} \quad \text{mg./24 hrs.}$$

(as previously described, see Introduction, Chapter II).

Example/

Example

If volume of the 24 hour sample of urine is 1000 ml. and radioactivity of 0.5 ml. after correction for quenching is 800 cpm then total count of 24 hour urine is 800 x 2 x 1000 = 1,600,000.

If activity of 3 ml. eluate is 4800 cpm and THE content of the same amount of eluate is 30 μg. then the specific activity of THE is

$$\frac{4800}{30} = 160 \text{ cpm}/\mu\text{g.} \quad \text{and}$$

$$\text{CPR} = \frac{1,600,000}{160} \times 10^{-3} = 10 \text{ mg.}/24 \text{ hrs.}$$

2. Estimation of 17-ketosteroids in urine (17-KS)

The method applied in this research is a modification of that described by James and deJong (1961).

Principle

After acid hydrolysis, urinary 17-ketosteroids were extracted by ethylene chloride and other acidic and phenolic steroids washed away by sodium hydroxide. The extract was dried and colour produced by a modified Zimmermann reaction; this was compared to the colour produced by a standard dehydroepiandrosterone (DHA) solution treated in the same way.

Colour reagent

a. m-dinitrobenzene: 1% solution in absolute ethanol.

b. Tetramethyl-ammonium hydroxide 25% aqueous solution (B.D.H.).

The colour reagent for the Zimmermann reaction was prepared by thoroughly mixing equal volumes of m-dinitrobenzene solution and tetramethyl-ammonium hydroxide solution immediately before use.

DHA standard:

a. Stock standard: 1 mg./ml. in absolute ethanol.

b. Working standards:

(1) 25 μg./0.2 ml. absolute ethanol.

(2) 50 μg./0.2 ml. absolute ethanol.

(3) 75 μg./0.2 ml. absolute ethanol.

(4) 100 μg./0.2 ml. absolute ethanol.

Procedure

24 hour urine samples were used, the volume measured and aliquots containing 4 - 5 drops of chloroform as preservative were kept in the deep freeze until required. Urine was well shaken and 10 ml. pipetted into clean/

clean 3/4 x 6" Quickfit test tube. 10 ml. distilled water were treated in the same way and served as a blank. 1 ml. concentrated hydrochloric acid was added and the contents of the tubes mixed and tubes placed in a boiling water bath in a fume cupboard for exactly 10 minutes. Tubes were then cooled and 10 ml. of ethylene chloride were added from a burette with ungreased tap. The tubes were then stoppered, sealed firmly with sellotape and contents mixed by gentle inversion (tumbling on a turntable) for 15 minutes at 45 r.p.m.

The ~~stopers~~ stoppers were removed and the tubes were centrifuged at 540 g. for 10 minutes. This should break any emulsion formed during the mixing. The top urine layer was carefully removed by suction with fine Pasteur pipette joined to a suction pump and 1 ml. N sodium hydroxide added. Tubes were restoppered, sealed and their contents mixed by tumbling on turntable for 7 minutes. Stoppers were removed and tubes recentrifuged at 540 g. for 10 minutes. The top alkaline layer was removed by suction and 1 ml. distilled water added. Tubes were stoppered and their contents mixed by tumbling for 7 minutes. Stoppers were removed and tubes centrifuged at 540 g. for 10 minutes. 2 gm. anhydrous sodium sulphate were added and tube shaken. An aliquot, 5 ml., of ethylene chloride extract was removed through cotton-wool into a clean dry 1/2 x 5" Quickfit test tube and evaporated to dryness under reduced pressure in a water bath at 80-90°C. 0.2 ml. absolute ethanol was added to each of the dry residues. Residues were dissolved by gentle but thorough mixing (stoppered tubes were warmed in hot water if necessary) 0.4 ml. of colour reagent was added and mixed. Tubes were allowed to stand in the dark at 25°C for 60 minutes./

minutes. 5 ml. of 70% aqueous ethanol was added and mixed.

"Specimen blank" was prepared for every batch of specimens substituting water for urine. 0.2 ml. of each working standard was pipetted into a standard specimen tube and test continued from the step of adding the colour reagent. "Standard blank" was prepared by processing 0.2 ml. absolute ethanol and test also continued from the step of adding the colour reagent. Optical densities of the specimen blank, standard blank, sample and standard tubes were determined using the S.P. 600 at wavelength 520 mμ. and at two other wavelengths equidistant on each side, viz. 440 mμ. and 600 mμ. (Allen, 1950), and the corrected final reading, i.e.

$$\text{O.D. at 520 m}\mu\text{.} - \frac{\text{O.D. at 440 m}\mu\text{.} + \text{O.D. at 600 m}\mu\text{.}}{2}$$

was calculated.

Blank standard values were subtracted from standard values and specimen blank values subtracted from unknown values.

<u>Calculation</u>

A standard graph was constructed by plotting the amount of standard per tube against corrected optical density and the corrected unknown values were read off from this graph, the result obtained was expressed as μg./0.2 ml. final extract. As only 10 ml. of urine were used in this estimation, the 17-KS in the 24 hour sample is:

$$\frac{\mu\text{g./0.2 ml. unknown}}{10} \times \text{total urine volume} \qquad \mu\text{g./24 hrs.}$$

$$\text{or} \quad \frac{\mu\text{g./0.2 ml. unknown}}{10} \times \text{total urine volume} \times 10^{-3} \qquad \text{mg./24 hrs.}$$

But/

But since only 5 ml. of the 10 ml. ethylene chloride extract were used, the final formula is:

$$\frac{\mu g./0.2 \text{ ml. unknown}}{10} \times \text{total urine volume} \times 10^{-3} \times \frac{10}{5} \quad \text{mg./24 hrs.}$$

3. Estimation of total 17-ketogenic steroids in urine (total 17-KGS)

The test used in this research is a modification of that described by Few (1961).

Principle

In urine samples, reduction by potassium borohydride of the C-17 ketone group of the pre-existing 17-KS and of the C-11 and C-20 ketone groups of the C-21 corticosteroids was followed by oxidation of all ketogenic steroids with sodium meta-periodate to the 17-KS, 11β-hydroxy-etiocholanolone and its 5α-isomer 11β-hydroxy-androsterone (see Introduction, Chapter II, and table 1). After alkaline hydrolysis the mixture was extracted with ethylene chloride, the extract washed and dried and the colour produced by Zimmermann reaction compared to the colour produced by a standard DHA treated in the same way.

The reagents and procedure were similar to those described by Few (1961) except that no chromatographic separation of 11-oxy and 11-deoxy total 17-KGS, ~~by chromatography~~ was attempted in this study and that tetramethyl-ammonium hydroxide was used in the colour reagent, as in estimation of 17-KS. Standard DHA solutions were prepared as in 17-KS method.

Procedure

24 hour urine samples were used. The total volume was noted and an aliquot of about 100 ml. containing 4 - 5 drops of chloroform as preservative was stored in the deep freeze till required. The urine was well shaken, 5 ml. pipetted into a clean $\frac{1}{2}$ x 5" Quickfit test tube. Few drops of ether were added to prevent frothing caused by the potassium borohydride,/

borohydride, of which 0.5 ml. was added (solution freshly made by dissolving 1 g. in 10 ml. 0.1 N sodium hydroxide). 5 ml. water were substituted for a blank. Tubes were stoppered and contents mixed then left either for 2 - 3 hours or overnight at room temperature to allow the reduction to take place. 0.25 ml. of 25% acetic acid was then added dropwise and tubes were shaken to mix their contents and allowed to stand for 15 minutes. For oxidation, 2 ml. of sodium meta-periodate solution (freshly made by dissolving 2 g. in 20 ml. distilled water) were added followed by 0.5 ml. of 1N sodium hydroxide. After thorough mixing, the pH was adjusted, if necessary,to 6.1 - 6.4 using narrow range pH papers (B.D.H., 5.5 - 8.5) externally and adding drop by drop 1N NaOH or 1N HCl where appropriate. The tubes were stoppered and placed in an incubator at 37°C for 1 hour. Hydrolysis was then carried out by adding 0.5 ml. of 2.5 N NaOH and mixing the contents of the tubes. Tubes were replaced in the incubator for 15 minutes.

Extraction with ethylene chloride was carried out as in the 17-KS method and 2.5 ml. of alkaline sodium dithionite solution (freshly prepared by dissolving 2.5 g. in 100 ml. 1.25 N sodium hydroxide) were added for purification. Extract was washed with 1 ml. of distilled water and anhydrous sodium sulphate was used for drying as in the 17-KS method. An aliquot, 5 ml. of solution,was removed, dried and the colour reaction proceeded as in the 17-KS method.

"Specimen blank", working standards and "standard blank" were prepared as in the 17-KS method and O.D. read in the S.P. 600, as previously described,/

described, at wavelengths 440, 520 and 600 mμ. Standard blank values were subtracted from standard values, and specimen blank values from unknown values.

Calculation

A graph was constructed by plotting the amount of standard per tube against corrected optical density. The corrected unknown was read off from this graph. The result obtained was expressed as μg./0.2 ml. final extract. Total 17-KGS in the 24 hour sample, therefore, equals

$$\frac{\mu g./0.2 \text{ ml. unknown}}{5} \times \text{total urine volume} \qquad \mu g./24 \text{ hrs.}$$

$$\text{or} \quad \frac{\mu g./0.2 \text{ ml. unknown}}{5} \times \text{total urine volume} \times 10^{-3} \qquad \text{mg./24 hrs.}$$

But since only 5 ml. of the 10 ml. ethylene chloride extract were used, the final formula is:

$$\frac{\mu g./0.2 \text{ ml. unknown}}{5} \times \text{total urine volume} \times 10^{-3} \times \frac{10}{5} \times 1.33 \qquad \text{mg./24 hrs.}$$

where 1.33 is a factor compensating for small extinction coefficient of 11β-hydroxy-etiocholanolone and 11β-hydroxy-androsterone as compared to DHA. This is because 11β-hydroxy- derivatives have only 74% of the chromogenicity of the corresponding 11-deoxy- compounds in the Zimmermann reaction (Few, 1961).

The presence of glucose in urine interferes with this estimation. In every specimen of urine, glucose was tested by Clinitest (Ames. Co.). If the glucose concentration exceeded 0.5 g./100 ml., the following procedure, recently recommended by the Medical Research Council Committee on Endocrinology/

Endocrinology (1963), was carried out in some of the specimens.

4 ml. of the urine were pipetted into a glass-stoppered test tube. 2 g. of ammonium sulphate were dissolved in the urine, which was then extracted four times with 4 ml. of an ether/ethanol (3/1) mixture. Extractions were made by shaking the two phases in the stoppered test tube. After allowing them to separate, the upper phase was transferred to an extraction tube. The combined ether/ethanol extract was evaporated on a water bath at 40°C in a current of nitrogen. 4 ml. of water were added to the residue in the extraction tube, and the normal procedure for determining total 17-KGS was then carried out as if the 4 ml. were urine.

4. Adrenocortical stimulation by ACTH

Initially ACTH gel (Crooks cortico-gel 40 I.U./ml.) was given by deep intramuscular injection, 60 I.U. every 12 hours for two days and once at 9:00 a.m. on the third day (i.e. 5 injections altogether) and cortisol production rate estimated by both blood and urine method on the third day, as usual. Urinary 17-KS and total 17-KGS were also estimated on the third day of ACTH stimulation. With this procedure, however, cortisol production rate obtained by the urine method was sometimes lower than that obtained by the blood method (see tables 11 and 12). It was thought at this stage that this discrepancy was due to the fact that the blood method involved specimens obtained only half an hour after the last injection of ACTH, i.e. during maximum stimulation of the adrenal cortex, while the urine method measured cortisol produced during the whole day. The interpretation of this observation, at this stage, was that stimulation of the adrenal cortex by this dosage of ACTH failed to produce a plateau of cortisol production lasting till the end of the third day. This observation was supported by the occasional finding of a lower urinary steroid output on the third day of adrenocortical stimulation than on the second day.

Accordingly, in the later part of the study, the dosage of ACTH was changed so that the patients received 60 I.U./12 hours for three days. Changing the dosage of ACTH, however, did not make any difference. The interpretation of these findings will be discussed later.

5. Pituitary-adrenocortical suppression by dexamethasone

This was carried out by oral administration of dexamethasone (Roussel) which was given in a dose of 0.5 mg. six hourly for two days and once at 9:00 a.m. on the third day. Cortisol production rate was estimated by both blood and urine methods on the third day, as usual. Again, urinary 17-KS and total 17-KGS were also estimated on the third day of dexamethasone suppression.

With this dosage, it was found that the cortisol production rate was so low that blood cortisol values, were below the sensitivity of the fluorometer. It was also found that most of the values of cortisol production rate after dexamethasone suppression obtained by the urine method were higher than those obtained by the blood method. The interpretation of this observation, at that stage, was that estimation of cortisol production rate by the blood method was carried out on the morning of the third day of adrenocortical suppression, only 30 minutes after administration of the last dose of dexamethasone, i.e. during the maximum suppression of the adrenal cortex, while the urine method measured the cortisol produced during the whole of the third day. This meant that suppression of the adrenal cortex by this dosage of dexamethasone was not being maintained till the end of the third day. This conclusion was supported, by the occasional finding of higher urinary steroid outputs on the third day of adrenocortical suppression than on the second day (see tables 17 and 18).

For these reasons it was decided, later on, to change the dosage of dexamethasone to 0.5 mg. six hourly for two days only, i.e. 8 doses altogether, and/

and to carry out the estimation of cortisol production rate, urinary 17-KS and total 17-KGS excretion on the second day of administration of the drug instead of the third day. Changing the dosage of dexamethasone did not make any difference. The interpretation of these findings will also be discussed later.

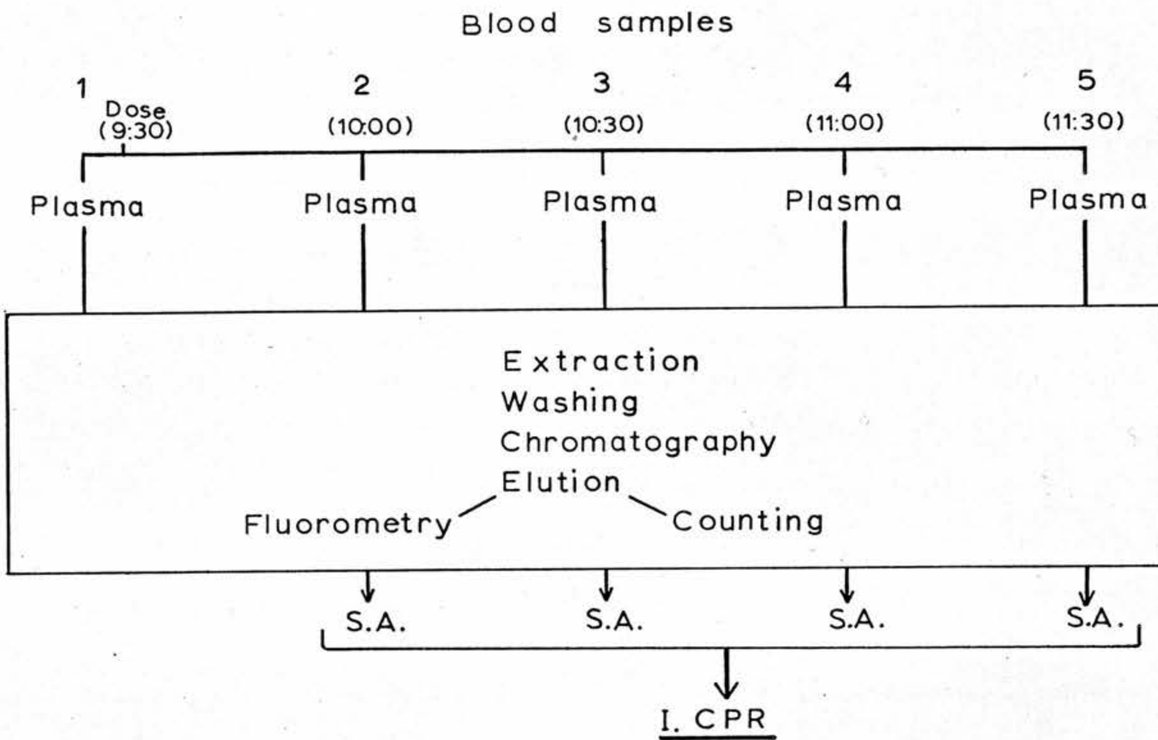


FIGURE 15 a : Simultaneous estimation of CPR, plasma cortisol and plasma NPB cortisol (in vitro)

1. Calculation of CPR

6. Simultaneous estimation of cortisol production rate, plasma cortisol and plasma-protein binding of cortisol.

A desire to estimate the degree of plasma-protein binding of cortisol as another index of adrenocortical function initiated the work which led to developing a method by means of which cortisol production rate, plasma cortisol and plasma-protein binding of cortisol could be simultaneously estimated, using 4-C^{14}-cortisol.

An *in vitro* method was tried first; radioactive cortisol was added to plasma collected before the C^{14}-cortisol was administered. Later on, an *in vivo* method was developed in which use was made of the plasma labelled by the injected dose to serve the three purposes.

a. *in vitro method*

Principle

A sample of blood was withdrawn and plasma separated. A trace amount of radioactive cortisol was added. A portion of this plasma was taken for total radioactivity counting. A second portion was subjected to ultrafiltration and the ultrafiltrate counted. A third portion, together with the plasma of the blood samples withdrawn after injection of a radioactive cortisol dose, was processed in the usual way, i.e. extraction, purification, chromatography, elution, radioactivity counting and fluorometric estimation of cortisol in the eluate.

Figure 15 (a and b) shows the main steps of this technique and the mode of calculating the results. Cortisol production rate was estimated, as previously described, from the specific activities of the blood samples withdrawn after injection of the dose. Cortisol was estimated fluorometrically in/

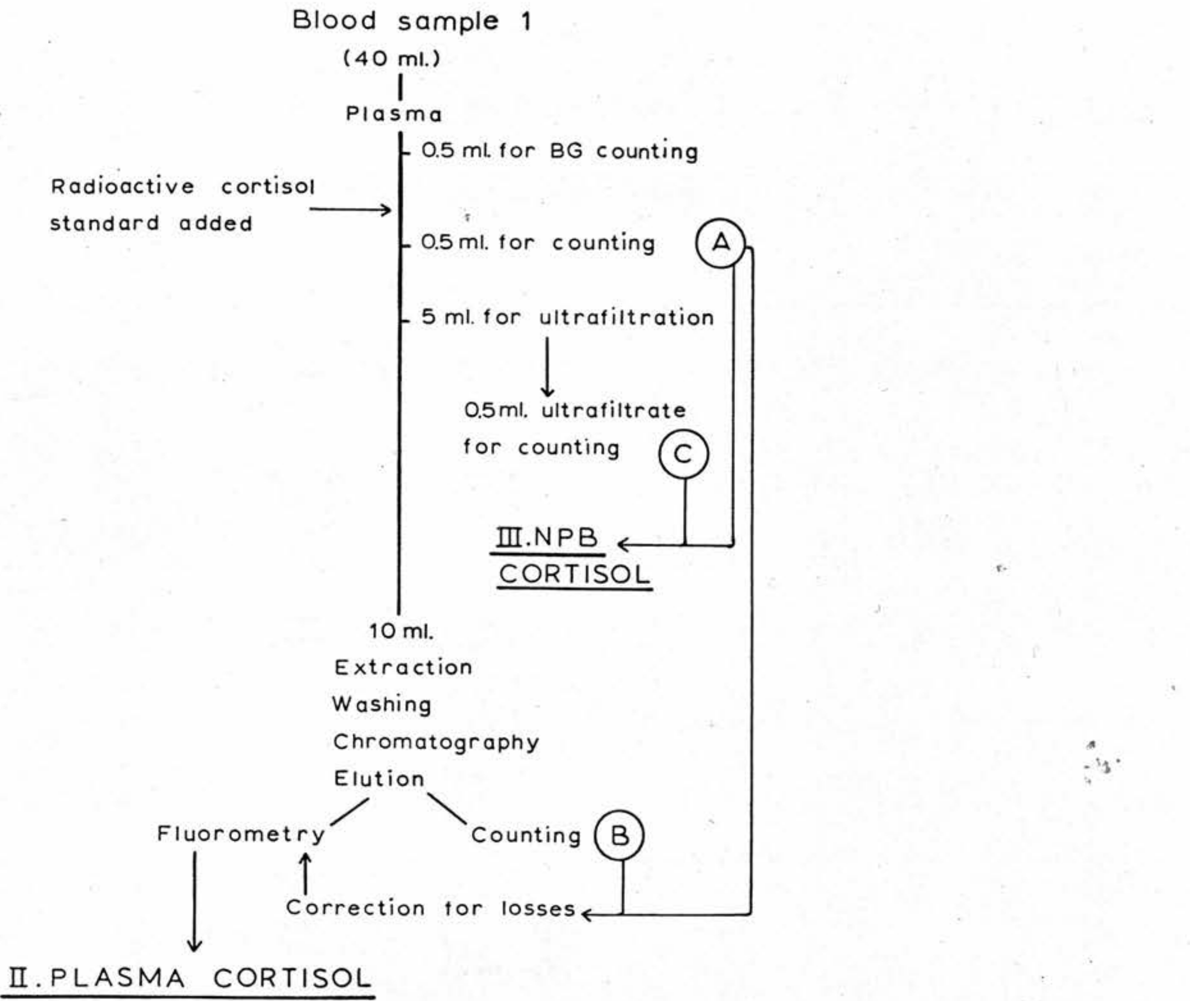


FIGURE 15 b: Simultaneous estimation of CPR, plasma cortisol and plasma NPB cortisol (in vitro)
2. Calculation of plasma cortisol and plasma NPB cortisol

in the purified extract of the pre-injection plasma, to which labelled cortisol was added, and the plasma cortisol obtained from this value by applying a correction, for the amount of cortisol lost during the extraction and purification. The correction factor was derived from estimation of the total radioactivity of the plasma and that of the purified extract.

The percentage of cortisol not bound to plasma proteins was calculated by comparing the total count of the ultrafiltrate to that of the original plasma.

Equipment

1. Ultrafiltration apparatus (see figure 16a). The apparatus used for ultrafiltration in this study was essentially similar to Toribara's ultrafiltration apparatus (Toribara, 1953) later used by Mills et al (1960) to study plasma-protein binding of cortisol, but much simpler.

The ultrafiltration apparatus used in this study consisted of:

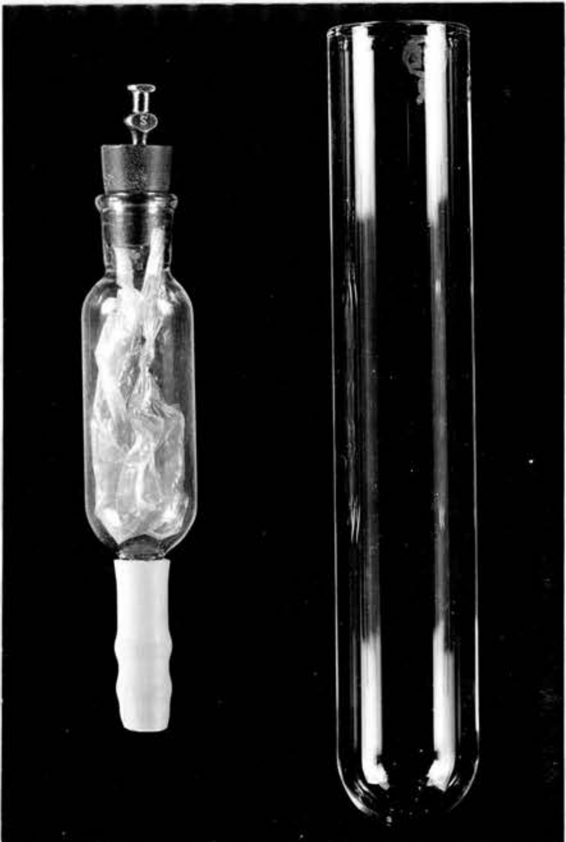
(a) Mercury levelling bulbs. These were glass tubes 7.5 cm. long and 2.5 cm. wide, with a narrow lower part, 3.5 cm. long and 0.75 cm. wide. Each was fitted with a rubber stopper at the upper end and a rubber tubing around and projecting for 0.5 cm. down the narrow end. The stopper was pierced by a thick syringe needle whose lower end was cut flush with the lower end of the stopper.

(b) 3 x 17 cm. centrifuge tubes (MSE - HR).

(c) Visking seamless regenerated cellulose tubing, size 18/32" with an average pore radius of 24 Ångstrom unit (H.M.C.)

The mercury ~~blub~~ bulb held inside the visking tubing, containing the plasma, and was placed inside the centrifuge tube where the ultrafiltrate collected. The stopper of the bulb was perforated to facilitate replacement of the ultrafiltrate/

(a)



(b)

(c)

Figure 16: Ultrafiltration apparatus.

ultrafiltrate by air from the centrifuge tube. The rubber tubing around the lower end of the bulb minimised friction between the glass surfaces of the latter and the bottom of the centrifuge tube and prevented either of them from getting broken during centrifugation. Parafilm and adhesive plaster were used to seal the centrifuge tube to prevent the ultrafiltrate from evaporating during centrifugation.

2. Carbon dioxide cylinder (5% in oxygen).

Reagents

The reagents were as for the estimation of cortisol production rate by the blood method, plus:

1. Hyamine solution; 1.5M: prepared by adding 33 ml. methanol to 75 g. Hyamine 10-X (Hopkins and Williams Ltd) to a flask and shaking vigorously till completely dissolved. This solution was kept in a dark place.

2. Internal standard: 5 ml. dilute dose solution (1:350) were further diluted with ethanol (1:1) so that 0.1 ml. of the final solution gave 400 cpm.

3. Dilute radioactive cortisol standard: prepared by diluting 0.1 ml. of the dose solution with 7 ml. saline; 0.05 ml. of this solution added to plasma (15 - 20 ml.) gave a total radioactivity of about 180 cpm/0.5 ml. plasma. This solution and the internal standard were kept at 4°C.

Procedure

The main steps of this procedure were the same as in the estimation of cortisol production rate by the blood method. Just before administration of the C^{14}-cortisol, 40 ml. of blood were withdrawn from the patient in a heparinised syringe and the plasma separated by centrifugation at 540 g. and 37°C. The C^{14}-cortisol was then administered. Four blood samples were withdrawn/

withdrawn at half hourly intervals and plasma separated from each as previously described.

0.5 ml. of the pre-injection plasma was pipetted in a counting jar for background counting. 0.05 ml. of the diluted dose solution was then added to the rest of the pre-injection plasma which was then mixed, by gentle inversion of the tube for few times, and incubated at 37°C for 10 minutes. Three portions of that plasma were used:

(a) A portion, 5 ml., was taken for ultrafiltration. This portion was pipetted in the visking tubing knotted at one end, and 5% carbon dioxide in oxygen was bubbled gently into the plasma for two minutes to maintain a relatively constant pH, utilising the buffer system of the plasma. The visking tubing was then held upright and gas driven out by pressing the walls of the tube between the thumb and index fingers. All the equipment necessary for ultrafiltration was warmed beforehand to 37°C in an incubator. The visking tube was then knotted at its upper end and introduced into the ultrafiltration tube so that it rested on the lower end (see figure 16b). The tube was stoppered and introduced into the centrifuge tube. The whole apparatus was again gassed with 5% carbon dioxide in oxygen and the centrifuge tube sealed with parafilm which was fixed with adhesive plaster (see figure 16c). The whole was introduced into a centrifuge bucket and spun at 540 g. for 5 hours at 37°C.

The centrifuge tube was taken out of the centrifuge bucket and left to cool to room temperature. The adhesive plaster and parafilm were then removed and the ultrafiltration tube taken out, leaving the crystal clear ultrafiltrate/

ultrafiltrate at the bottom of the centrifuge tube. 0.5 ml. of the ultrafiltrate was removed by a pipette into a jar for radioactivity counting.

(b) A second portion, 0.5 ml., of this plasma was pipetted into a counting jar and used for the estimation of total radioactivity of the plasma.

(c) A third portion, 10 ml., and all the plasma of the four post-injection samples were subjected to the same procedure, i.e. extraction, purification, chromatography, elution, radioactivity counting and fluorometric estimation of cortisol in the eluate.

Plasma and ultrafiltrate were counted in a way which was different from that described for counting urine or eluates. This was because plasma did not blend with the phosphor and the incorporation of alcohol as in counting urine or plasma eluates rendered the solution very turbid. But plasma can be blended with the phosphor if it is first dissolved in hyamine solution, 1.5M. Accordingly three counting jars were prepared as follows:

	Non-radioactive plasma ml.	Radioactive plasma ml.	Ultrafiltrate ml.	Hyamine solution ml.	Toluene phosphor ml.
Background	0.5	-	-	2	9.5
Plasma	-	0.5	-	2	9.5
Ultrafiltrate	-	-	0.5	2	9.5

Plasma, or ultrafiltrate, to be counted was dissolved thoroughly in hyamine solution before adding the phosphor. The counting jar was then kept in the dark in a petri dish containing tap water for 10 minutes to bring it to the temperature of the castle and then housed in the latter for another 10 minutes/

minutes before counting started. Background was counted till 4,000 counts were recorded and all active samples counted till 10,000 counted were recorded.

Because plasma is invariably not clear, quenching occurs as with urine. The amount of quenching varies with the different plasmas. Allowance for quenching was made as follows: after counting the plasma, 0.1 ml. of the internal standard was added and the (plasma + standard) jar counted again. The plasma activity was corrected for quenching by multiplying the observed value by the ratio of actual/observed activity of the standard.

The validity of correction for quenching was tested by an experiment in which a known amount of activity was added to 8 different plasma samples and the observed counts were compared to those corrected after using a radioactive standard.

Table (8)

Validity of the correction method for quenching of plasma

Plasma Sample (No.)	Added radioactivity (cpm)	Observed radioactivity (cpm)	Corrected radioactivity (cpm)	Efficiency (%)
1	250	238	259	103.6
2	250	227	247	98.8
3	250	236	255	102.0
4	250	218	242	96.8
5	250	221	246	98.3
6	250	214	252	100.7
7	250	232	250	100.0
8	250	214	257	102.9

96.8 - 103.6

The results (table 8) show clearly that not only is this correction necessary but also that it is very reliable. Such correction, however, was not/

not found necessary with the ultrafiltrate, which was always clear, i.e. could not produce any quenching; the observed activity of ultrafiltrates in cpm were directly used in the calculations.

Counts of the background were almost always 100 cpm (ranged from 96 - 104 cpm), those of the active samples, after subtraction of the background count, were about 180 cpm for the plasma, 12 - 28 for the ultrafiltrate and 400 for the internal standard.

The internal standard was prepared such that the activity of the combined standard and plasma was always more than double the activity of the plasma in order to determine the amount of quenching correctly.

After dexamethasone administration counts of the ultrafiltrate in the great majority of cases approached the background count, thus rendering their value questionable. This problem could not be overcome without adding much higher amounts of radioactive cortisol to the plasma. The amount of radioactive cortisol which was used in this technique raised the plasma cortisol by less than 0.1 µg./100 ml. To obtain comparable counts after dexamethasone would mean adding measureable amounts of cortisol, which would significantly affect the results of cortisol estimation and most probably also affect the proportion of bound to unbound cortisol, thus invalidating the whole test.

Calculation

1. Size of the miscible pool, the biological half clearance time, the rate of turnover and production rate of cortisol. These were calculated, as previously described, from the specific activities of the purified extracts of the four post-injection samples.

2./

2. Plasma cortisol

(a) Correction for quenching:

Suppose the actual activity of the internal standard added to the pre-injection plasma was 400 cpm, the observed activity of 0.5 ml. plasma, with added C^{14}-cortisol, was 200 cpm and that of the plasma plus internal standard was 520 cpm.

The observed activity of the internal standard, therefore, is 520 - 200 = 320 cpm.

The activity of plasma corrected for quenching equals

$$\text{observed activity of plasma} \times \frac{\text{true activity of standard}}{\text{quenched activity of standard}}$$

which in this example equals

$$\frac{200 \times 400}{320} = 250 \text{ cpm/0.5 ml. plasma}$$

$$\equiv 5000 \text{ cpm/10 ml. plasma}$$

(b) Correction for losses during extraction and purification:

Suppose the final extract, which corresponds to 10 ml. plasma has a total activity of 2500 cpm and contains 0.6 μg. of cortisol. The original plasma has an activity, corrected for quenching of 5000 cpm/10 ml. The recovery is therefore

$$\frac{2500}{5000} \times 100 \% = 50\%$$

The plasma cortisol, uncorrected for losses is 0.6 μg./10 ml. (≡ 6 μg./100 ml.). The corrected plasma cortisol

$$= 6 \times \frac{100}{50} \text{ μg./100 ml.}$$

$$= 12 \text{ μg./100 ml.}$$

Correction/

Correction for losses in this way is based on the assumption that the added radioactive cortisol mixes homogeneously with the non-radioactive endogenous one, so that losses of the radioactive cortisol should also represent losses of the endogenous one.

3. Non-protein-bound cortisol (NPB cortisol)

The number of cortisol molecules per unit volume of ultrafiltrate equals the number of NPB cortisol per unit volume of plasma water. In theory, the percentage of NPB cortisol equals the activity in 0.5 ml. ultrafiltrate expressed as a percentage of the activity of the same volume of the original plasma, with a correction for the protein content of plasma.

Suppose the activity of the ultrafiltrate was 25 cpm/0.5 ml. The activity of the original plasma, corrected for quenching, was 250 cpm/0.5 ml. plasma; this corresponds to an activity of $250 \times \frac{100}{93}$ cpm/0.5 ml. plasma, assuming the plasma water content to be 93%. The percentage of NPB cortisol is then

$$25 \times \frac{93}{250 \times 100} \times 100 \; \% \qquad = 9.3 \; \%$$

The amount of non-protein-bound cortisol equals

% NPB cortisol x plasma cortisol

i.e. $\frac{9.3}{100} \times 12$ µg./100 ml.

= 1.12 µg./100 ml.

In this study, data for plasma protein values were not available. Percent NPB cortisol was calculated directly from activities of original plasma and ultrafiltrate. Thus, using the figures of the previous example:

$$\% \text{ NPB cortisol} = \frac{25}{250} \times 100 = 10 \; \%$$

and the amount of NPB cortisol $= \frac{10}{100} \times 12 = 1.2$ µg./100 ml.

Because/

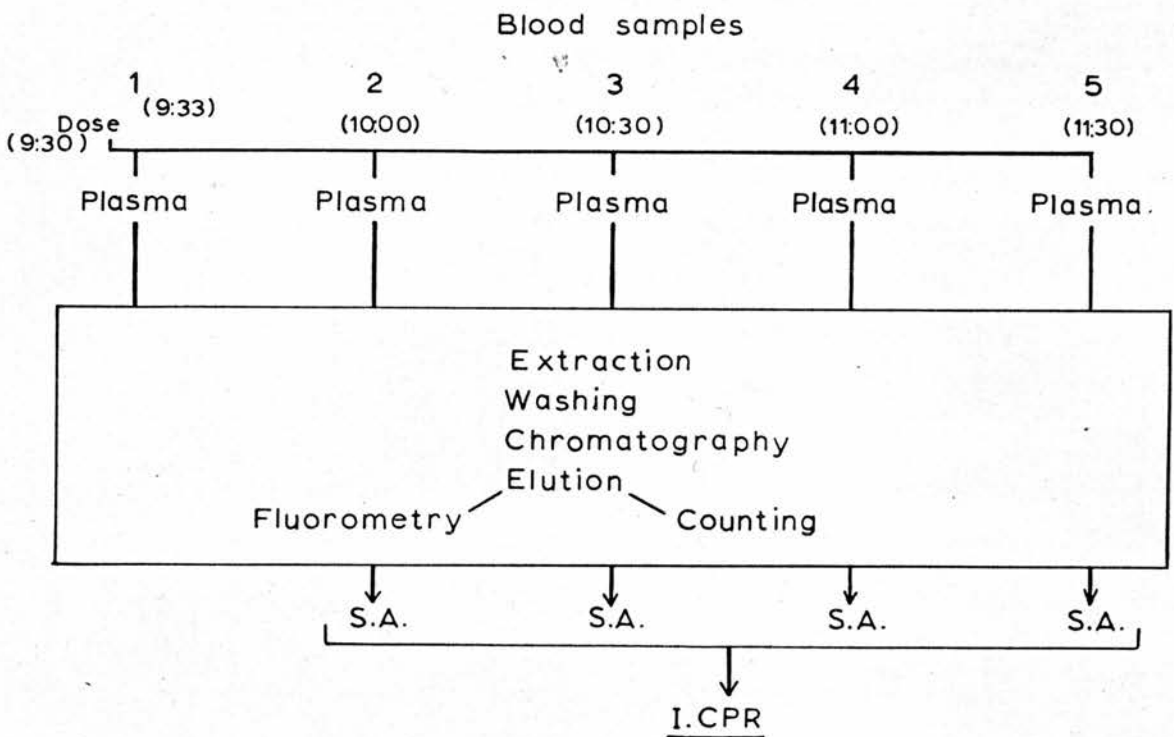


FIGURE 17 a : Simultaneous estimation of CPR, plasma cortisol and plasma NPB cortisol (in vivo)

1.Calculation of CPR

Because the majority of cortisol in plasma is bound to proteins, the error resulting from not including a correction for the volume of plasma proteins is likely to be slight. Mills (1961 a), using the ultrafiltration technique for estimation of NPB cortisol, did not find it necessary to apply such a correction.

The theory behind estimation of NPB cortisol in this way is based on the assumption that radioactive cortisol added to plasma gets in equilibrium with the endogenously produced cortisol, protein-bound and non-protein-bound, and that the amount added is too small to affect the cortisol level, or equilibrium, to any significant extent. Radioactive cortisol used in this study was of very high specific activity (60 μc./mg.). Accordingly, the amount added only raised the plasma level by less than 0.1 μg./100 ml. Such a very small amount was assumed not to be able to affect the equilibrium between protein- and non-protein-bound cortisol.

b. in vivo method

Principle

This is the same as in the in vitro method. A trace amount of C^{14}-cortisol was injected intravenously and 5 blood samples withdrawn. The first of these samples was used to obtain the plasma cortisol and the plasma NPB cortisol values (see figure 17 a and b)

Equipment and reagents

The same as in the in vitro method.

Procedure

Again, the main steps of this procedure were essentially the same as in the/

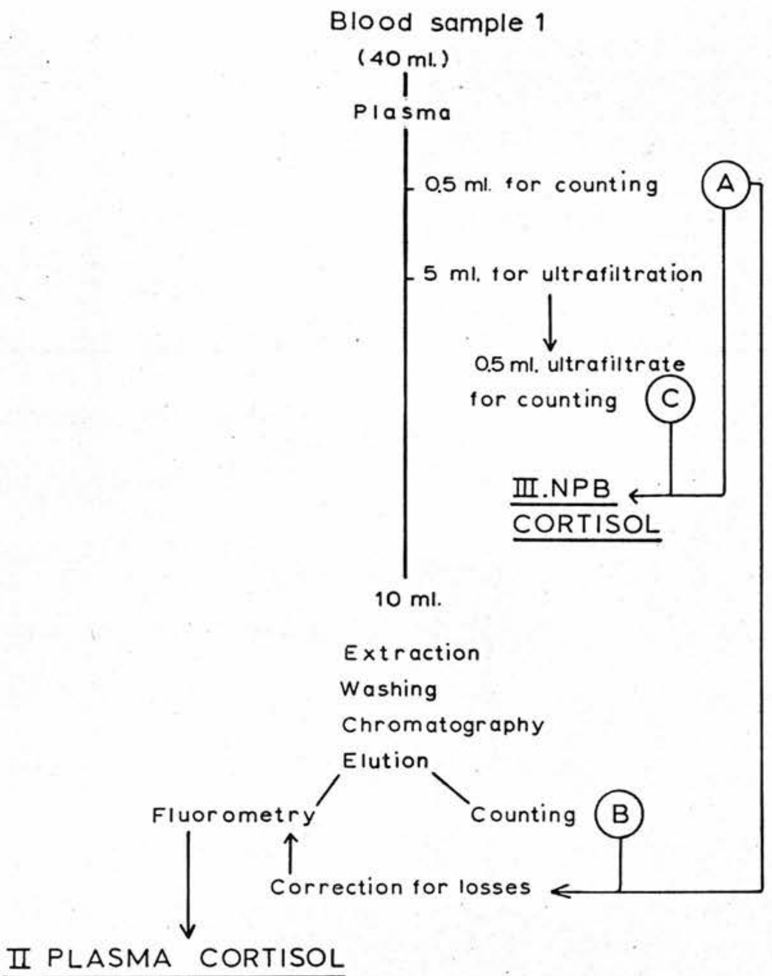


FIGURE 17 b: Simultaneous estimation of CPR, plasma cortisol and plasma NPB cortisol (in vivo)

2. Calculation of plasma cortisol and plasma NPB cortisol

the *in vitro* method. 1 µc. 4-C^{14}-cortisol was used at first as one dose, but as has been discussed, the activity of the ultrafiltrate was a relatively small percentage of the total activity of the plasma. This meant that the counting of the ultrafiltrate was so near that of the background, that the accuracy of counting the ultrafiltrate and accordingly of calculating the NPB cortisol was too low. Accordingly, the dose was increased to 2 µc. and much better results, well within the reliability margin, were obtained.

Just before administration of the dose, 2 ml. blood were withdrawn from the patient in a heparinised syringe and plasma separated. 0.5 ml. of this plasma was used as background in counting the active plasma.

The dose was administered at 9:30 a.m. as described before. At half hourly intervals, starting 3 minutes after administration of the dose, 5 blood samples were withdrawn into heparinised syringes, as previously described, with the exception that 40 ml. of blood were collected for the first sample instead of the usual 20 ml. Plasmas were immediately separated.

10 ml. plasma of the first sample, and all plasma of the other samples were extracted, washed, chromatographed and the specific activities of their cortisol content estimated as previously described.

From the remaining plasma of the first sample a 0.5 ml. portion was pipetted into a counting jar and used for the estimation of the total activity of the plasma.

A third portion of 5 ml. was used for ultrafiltration as described in the *in vitro* method. Again, all the equipment necessary for ultrafiltration was warmed beforehand to 37°C in an incubator.

Counts/

Counts of the background were almost always 100 cpm. (ranged from 96 to 104), those of the radioactive samples, after subtraction of the background count were: 178 - 268 cpm for the plasma, 400 cpm for the internal standard and 16 to 40 cpm for the ultrafiltrate.

After dexamethasone suppression, counts of the ultrafiltrate, again, approached that of the background. This problem could not be overcome without administering a large dose of radioactive cortisol which would result in significant increase of plasma cortisol and probably also in disturbance of the equilibrium between protein-bound and NPB cortisol.

Calculation

1. The size of body miscible pool, biological half clearance time, rate of turnover and production rate of cortisol. These were calculated from the specific activities of the second, third, fourth and fifth blood samples and the application of the isotope dilution expressions, as previously described.

2. Plasma cortisol and NPB cortisol were estimated as in the *in vitro* method, with the only difference that figures obtained from the first plasma sample withdrawn after injection of the C^{14}-cortisol in the *in vivo* method were used instead of those of the pre-injection plasma to which radioactive cortisol was added in the *in vitro* method.

The theory behind this technique is based on the following assumptions:

(a) that the amount of radioactive cortisol administered to the patient should be so small that it will not significantly affect either the plasma cortisol or the proportion of protein-bound to NPB cortisol. This was verified in the present study by administering a trace amount of cortisol (about 30 μg.).

(b)/

(b) that the administered cortisol will get in equilibrium with endogenous cortisol (protein-bound and NPB). Binding of the injected radioactive cortisol occurred rapidly after injection, in fact more efficiently than in the *in vitro* method. This will be evident later, from comparison of the results of the *in vitro* and *in vivo* methods.

(c) that the radioactivity contained in the plasma withdrawn after injection of the C^{14}-cortisol should be due to cortisol and not to its radioactive metabolites. This was verified by collecting the first sample 3 minutes after injection of the radioactive dose; the amount of radioactive metabolites formed in this very short period should not be significant.

(d) that the three-minutes' interval between starting the injection and collecting the first blood sample is sufficient to let the radioactivity circulate in the body and get back to the site of venepuncture. This is indeed the case, since the arm-to-arm circulation takes only about 21 seconds (Bard, 1961).

Other chemical tests

Besides the previously mentioned tests for the direct assessment of adrenocortical function, other chemical tests were performed for every case whenever possible. These tests include:

(1) Estimation of creatinine excretion (AutoAnalyzer) for correlation of the total body musculature to the adrenocortical activity.

(2) Tests to exclude renal insufficiency, viz. creatinine clearance (AutoAnalyzer), blood urea (AutoAnalyzer) and urinary proteins (Albustix, Ames Co.).

(3) Tests to detect diabetes, viz. glucose tolerance tests (AutoAnalyzer), urinary glucose (Clinitest, Ames Co.) and urinary ketones (Acetest, Ames Co.)

(4) Tests to detect any electrolyte disturbance or alkalosis, viz. serum or plasma sodium (Flame photometer, EEL), potassium (Flame photometer, EEL), bicarbonate (AutoAnalyzer),calcium and phosphate.

(5) Tests to exclude liver dysfunction, viz. serum bilirubin, serum glutamic-pyruvic transaminase, alkaline phosphatase and thymol turbidity.

B. Other laboratory investigations

These included tests of additional diagnostic help such as:

(1) Histological examination of adrenal glands removed from patients with Cushing's syndrome treated by adrenalectomy.

(2) Haematological examination (haemoglobin, packed cell volume, mean corpuscular haemoglobin concentration, total and differential white cell count and platelet count).

(3) X-ray of the chest to detect any enlargement of the heart.

(4) X-ray of the skeleton to detect osteoporosis.

(5) X-ray of the skull for any widening of the sella turcica.

(6) Electrocardiography.

(7) Medical photography (anterior and lateral views).

(8) Estimation of total body water from which the lean cell mass and total body fat could be calculated and correlated to the adrenocortical function.

Estimation of total body water was carried out in the Department of Medical Physics of the Royal Infirmary of Edinburgh and was performed by the isotope dilution methods described by Langham et al (1956) and Cooper et al (1958).

Estimation of total body water required the administration of tritiated water with an activity of 500 μc. As the counter used for C^{14}-counting was not adjusted to exclude counts due to tritium, it was felt more convenient to postpone estimation of the total body water of each patient to the end of the study using C^{14}-cortisol.

From/

From estimation of the total body water, the body lean cell mass (LCM) and body fat cen be worked out from the following equations (Osserman et al, 1950):

(1) % LCM = Ak

where % LCM is the total body lean cell mass as a percentage of total body weight.

A is the total body water as a percentage of the total body weight.

k is a constant ($\frac{1.000}{0.718}$ or 1.393).

(2) % Fat = 100 - % LCM

where % Fat is the total body fat as a percentage of total body weight.

Accordingly:

Total body LCM (Kg.) = % LCM x Total body weight (Kg.) and

Total body fat (Kg.) = % Fat x Total body weight (Kg.)

C. Clinical investigations

Each patient was thoroughly assessed as follows:

(1) In taking the family history patients were questioned particularly about obesity, diabetes or any other endocrine disease.

(2) The history of the presenting condition and its duration, including any menstrual disturbance in females.

(3) Clinical examination

(a) Face, hair, skin (with particular reference to acne, bruising and oedema).

(b) Muscle development and power.

(c) Genitals and their development.

(d) Assessment of the degree of obesity and, if present, the patient was asked about its duration and a note made of its distribution.

(e) Assessment of the extent of cutaneous striae, if present, their colour, distribution and duration.

(f) Examination of the blood pressure.

The surface area of each patient was calculated from the height and weight as suggested by DuBois and DuBois (1916) from the formula:

$$S.A. = W^{0.425} \times H^{0.725} \times 0.007184$$

where S.A. is surface area in square meters.

W is weight in kilograms.

H is height in centimeters.

Part III

Results

A. Cortisol production rate and steroid excretion

1. Cortisol production rate (blood method), control estimations.

Table 9 shows the results of estimation of cortisol production rate by the blood method in the four groups under basal conditions (see also figure 18). The results reveal the following:

(a) The miscible pool

There is overlap between the ranges found in the four groups. Statistical analyses of these results (see table 28) show that the increase of the mean in the patients with Cushing's syndrome above the mean of either the normal or obese group is highly significant.* No significant difference exists between the means of the normal and obese groups nor between the 'Cushingoid' and other groups. The range in the 'Cushingoid' group is wide.

These results are in agreement with those reported by Hoet et al (1961) who reported mean values of 1.54 mg. for normal subjects, 1.27 mg. for obese subjects and a range of 2.3 - 7.3 mg. for patients with Cushing's syndrome. Peterson and Wyngaarden (1956) reported a lower range for normal subjects, namely 2.4 mg.

(b) The biological half clearance time ($t\frac{1}{2}$) and the rate of turnover

There is an overlap between the ranges found in the four groups and no significant differences exist between the means of the normal, Cushing's and obese group. Only the difference between the means of the Cushing's and 'Cushingoid' group is significant.

Figures reported by other workers for the biological half clearance time of normal subjects are; 60 - 90 minutes (Peterson et al, 1955), 50 - 130 minutes/

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* Throughout part III of this thesis:
not significant = statistically not significant ($P > 0.05$)
significant = statistically significant ($0.05 > P > 0.01$)
highly significant = statistically highly significant ($P < 0.01$)

Table (9)

Cortisol production rate (blood method)

Control estimations

Case	Age (yrs.)	Sex	Miscible pool (mg.)	$t_{\frac{1}{2}}$ (min.)	Rate of turnover (pool/hr)	Production rate (mg./24 hr.)
1. Normal group						
1. B.A.*	29	M	1.2	63	0.66	20.4
2. R.B.**	65	M	3.1	108	0.38	30.0
3. W.J.**	35	M	1.3	75	0.55	17.5
4. W.H.**	55	M	2.6	72	0.50	31.2
5. M.L.**	42	F	1.6	72	0.57	21.8
6. M.H.**	35	F	1.2	60	0.65	28.8
7. M.R.**	23	F	1.4	54	0.77	26.9
Range	23-65		1.2-3.1	54-108	0.38-0.77	17.5-31.2
Mean	40.5		1.8	72	0.58	25.2
S.D.			0.74		0.11	5.3
S.E.			0.28		0.04	2.0
2. Cushing's group						
a. Operated upon						
10. J.A.*	55	M	7.4	42	0.99	177.6
11. J.T.*	17	F	2.5	45	0.93	54.7
12. M.C.*	36	F	2.9	75	0.54	37.9
b. Not operated upon						
17. C.M.*	18	F	2.6	78	0.54	34.1
Range	17-55		2.5-7.4	42-78	0.53-0.99	34.1-177.6
Mean	31.5		3.9	60	0.75	76.1
S.D.			2.38		0.25	68.3
S.E.			1.19		0.12	34.1

Table (9) contd

Case	Age (yrs)	Sex	Miscible pool (mg.)	t½ (min.)	Rate of turnover (pool/hr)	Production rate (mg./24 hr.)
3. 'Cushingoid' group						
18. A.D.*	19	F	2.1	56	0.74	37.0
19. W.C.*	27	M	2.1	92	0.45	23.0
20. D.M.**	21	M	4.7	105	0.40	45.6
21. T.M.*	18	M	2.6	88	0.50	31.2
22. C.A.*	26	F	1.2	144	0.29	8.6
23. D.S.**	34	F	2.0	105	0.39	18.7
24. M.K.**	15	F	2.1	63	0.60	30.2
25. D.Y.**	32	F	2.2	81	0.51	26.4
Range	15-34		1.2-4.7	56-144	0.29-0.74	8.6-45.6
Mean	24.0		2.3	91.8	0.49	27.6
S.D.			1.00		0.14	11.2
S.E.			0.35		0.05	3.9
4. Obese group						
26. A.M.*	34	M	0.8	50	0.84	18.0
27. R.A.*	12	M	0.9	34	1.20	25.9
28. T.A.**	17	M	1.3	60	0.70	22.1
29. C.S.*	14	F	3.3	110	0.37	28.8
30. H.L.*	31	F	1.9	100	0.41	18.7
31. A.I.**	24	F	2.3	108	0.38	21.4
32. C.R.*	20	F	1.0	78	0.53	13.2
33. M.S.*	14	F	2.3	75	0.55	30.5
Range	12-34		0.8-3.3	34-110	0.37-1.20	13.2-30.5
Mean	20.8		1.7	76.9	0.62	22.3
S.D.			0.85		0.28	5.8
S.E.			0.30		0.10	2.0

* Dose 1 μc.
** Dose 2 μc.

minutes (Peterson and Wyngaarden, 1956), 81 minutes (Slaunwhite and Sandberg, 1961) and 78 minutes (Tait et al, 1961). Hoet et al (1961) comparing normal subjects, obese subjects and patients with Cushing's syndrome reported a mean of 58 minutes in the normal group (0.88 ± 0.34 pool/hr.), 61 minutes in the obese group (0.72 ± 0.19 pool/hr.) and 45 - 66 minutes in the patients with Cushing's syndrome (0.62 - 0.93 pool/hr.).

(c) Cortisol production rate

The normal and obese subjects are narrowly separated from the patients with Cushing's syndrome; all normal and obese subjects had values below 31.2 mg./24 hrs. and all those with Cushing's syndrome had values above 34.1 mg./24 hrs. The increase in the mean of the patients with Cushing's syndrome above the mean of either the normal or the obese group is significant. The range of the 'Cushingoid' group is very wide. Difference between the means of the normal and obese groups is not significant.

The range and mean of the normal group is slightly higher than those reported by Peterson and Wyngaarden (1956), namely 12.0 - 29.0 and a mean of 21.0 mg./24 hrs. Hoet et al (1961), however, reported comparable figures; namely 1.09 ± 0.26 mg./hr. (≡ 26.2 ± 6.2 mg./24 hrs.) in a normal group, which was not significantly different from the mean of an obese group, 0.97 ± 0.31 mg./hr. (≡ 23.3 ± 7.5 mg./24 hrs.), while a third group of patients with Cushing's syndrome due to adrenal hyperplasia were in the range of 2.1 - 4.5 mg./hr. (≡ 50.4 - 108.0 mg./24 hrs.) which was significantly higher than both the normal or obese group. Similarly, Prunty (1961) reported a range of 39 - 420 mg./24 hrs. in 12 cases of Cushing's syndrome.

2. Cortisol production rate (urine method) and urinary steroid excretion, control estimations

Table 10 shows the results of estimation of cortisol production rate by the urine method and the urinary excretion of 17-ketosteroids and total 17-ketogenic steroids under basal conditions (see also figures 19-21). In those cases in which the blood method was also performed, all tests were carried out on the same day to allow comparison between the results of the four tests that were made.

(a) Percent of radioactive cortisol dose excreted in urine in the first 24 hrs.

Of all the 30 subjects studied, 27 excreted more than 70% of the radioactive dose, 23 more than 75% and 18 more than 80%. No significant differences exist between the means of all four groups (see table 28).

The ranges and means for the excretion of radioactivity after 24 hrs. were of the same order as those reported by other workers after administration of labelled cortisol for normal subjects viz. 70 - 82% (Peterson et al, 1955), 75 - 85% (Cope and Black, 1958 a), 68 - 95% (Cope and Black, 1958 b), 79 - 94% (Romanoff et al, 1961), 83 - 96% (Flood et al, 1961) and 90% (Layne et al, 1962). In a more detailed study, Hellman et al (1954) observed that 15% of the radioactive dose was excreted after 1 - 2 hours, 56% after 6 hours and 72 - 84% at the end of 24 hours. Similarly, Migeon et al (1956 a) observed excretion of 2 - 14% in 2 hours, 25 - 33% in 4 hours, 40 - 60% in 8 hours, 60 - 70% in 14 hours and 70 - 83% in 24 hours and 80 - 90% in 48 hours.

Gray et al (1962) observed excretion of 86% in a case of Cushing's syndrome against a range of 65 - 86% in normal subjects and Schteingart et al (1963)/

Table (10)

Cortisol production rate (urine method) and urinary steroid excretion

Control estimation

Case	Age (yrs)	Sex	Dose excretion first 24 hr (%)	S.A. THE (cpm/μg)	Production rate (mg./24hr.)	17-KS (mg./24hr)	Total 17-KGS (mg./24hr)
1. Normal group							
8. T.T.*	72	M	73.0	20.0	26.6	11.0	7.4
9. S.F.*	42	M	79.6	16.4	29.0	11.2	7.3
2. R.B.**	65	M	84.1	95.3	23.7	7.0	19.0
3. W.J.**	35	M	76.5	151.8	13.5	5.0	22.0
4. W.H.**	55	M	-	-	-	10.0	18.0
5. M.L.**	42	F	82.7	187.8	11.6	13.0	14.0
6. M.H.**	35	F	72.6	142.3	12.9	4.0	10.0
7. M.R.**	23	F	84.0	101.1	22.4	10.0	18.0
Range	23-72		72.6-84.1		11.6-29.0	4.0-13.0	7.3-22.0
Mean	46.1		78.9		20.0	8.9	14.5
S.D.			4.9		7.1	3.2	5.6
S.E.			1.8		2.7	1.2	2.1
2. Cushing's group							
a. Operated upon							
10. J.A.*	55	M	80.0	5.6	107.0	18.7	54.9
13. J.M.*	31	M	99.0	16.1	36.5	22.5	35.3
14. G.F.	27	M	-	-	-	32.7	45.4
11. J.T.*	17	F	85.9	10.9	62.2	14.2	15.8
12. M.C.*	36	F	78.0	37.7	24.6	10.2	21.8
15. H.T.*	21	F	87.9	16.7	25.2	14.5	14.2
16. E.S.*	22	F	49.0	11.6	33.8	11.0	12.7
b. Not operated upon							
17. C.M.*	18	F	92.0	23.6	44.7	10.8	29.0
Range	17-55		49.0-99.0		24.6-107.0	10.2-32.7	12.7-54.9
Mean	28.4		81.6		47.7	16.8	28.6
S.D.			16.0		29.1	7.6	15.5
S.E.			6.0		12.0	2.7	5.4

Table (10) contd

Case	Age (yrs)	Sex	Dose excretion first 24 hr. (%)	S.A. THE (cpm/µg)	Production rate (mg./24hrs)	17-KS (mg/24hr)	Total 17-KGS (mg/24hr)
3. 'Cushingoid' group							
18. A.D.*	19	F	80.8	23.3	33.2	2.3	30.6
19. W.C.*	27	M	69.2	29.2	22.1	10.3	20.7
20. D.M.**	21	M	88.9	39.3	41.2	30.0	30.0
21. T.M.*	18	M	87.9	40.4	26.8	7.4	28.9
22. C.A.*	26	F	78.0	28.0	13.4	16.7	17.5
23. D.S.**	34	F	73.2	132.7	15.0	8.0	13.0
24. M.K.**	15	F	76.7	72.6	27.4	12.4	16.9
25. D.Y.**	32	F	82.8	120.9	18.7	5.0	13.0
Range	15-34		69.2-88.9		13.4-41.2	2.3-30.0	13.0-30.6
Mean	24.0		79.6		24.0	11.5	21.3
S.D.			6.8		9.4	8.6	7.4
S.E.			2.4		3.3	3.0	2.6
4. Obese group							
26. A.M.*	34	M	39.6	22.1	19.2	-	-
27. R.A.*	12	M	81.3	49.3	15.5	8.6	9.5
28. T.A.**	17	M	89.8	152.2	15.8	6.0	22.0
29. C.S.*	14	F	88.4	34.5	27.7	6.7	13.0
30. H.L.*	31	F	85.8	47.1	17.0	8.6	16.3
31. A.I.**	24	F	80.8	59.6	22.5	5.0	16.0
32. C.R.*	20	F	87.1	52.7	17.8	9.2	11.7
33. M.S.*	14	F	83.5	40.2	19.6	8.2	12.9
Range	12-34		39.6-89.8		15.5-27.7	5.0-9.2	9.5-22.0
Mean	20.8		79.5		20.8	7.5	14.5
S.D.			16.4		4.0	1.5	4.0
S.E.			5.8		1.4	0.6	1.5

* Dose 1 µc.
** Dose 2 µc.

(1963) comparing normal subjects, obese subjects and patients with Cushing's syndrome found that the means of excretion were 84.1, 83.1 and 85.0% for these three groups respectively, also with no significant difference between them. It is clear, therefore, that cortisol metabolism is, firstly, very rapid and, secondly independent of the state of adrenocortical function.

(b) Cortisol production rate (CPR)

The levels reached by two of the normal and one of the obese subjects overlap with those reached by two patients with Cushing's syndrome. The range of the 'Cushingoid' group is again wide. The mean of the group of patients with Cushing's syndrome is significantly higher than that of the normal or the obese group. The difference between the means of the normal and obese groups is not significant.

The results obtained by the urine method are mostly lower than those of the blood method especially in the normal group (see table 12 a). The reason could be that the urine method measured the production rate during 24 hours, while the blood method measured it between 9:30 and 11:30 a.m. in the morning, a period known to be associated with increased adrenal activity relative to the rest of the day.

Similar figures of CPR by the urine method have been reported by other workers for normal subjects; namely, 4.9 - 25.3 mg./24 hrs. (Cope and Black, 1958 b), 4.2 - 24.0 mg./24 hrs. (Cope and Black, 1958 a), 15.9 - 31.1 mg./24 hrs. (Romanoff et al 1961), 16.1 ± 0.8 mg./24 hrs., mean and S.E., (Layne et al, 1962) and 9.5 - 31.3 mg./24 hrs. (James and Caie, 1964).

Prunty (1961) reported a range of 41 - 111 mg./24 hrs. in a group of patients with Cushing's syndrome, and Brooks et al (1963) reported a range of 32.0/

32.0 - 96.0 mg./24 hrs. for cases of Cushing's syndrome due to adrenal hyperplasia and 39.0 - 82.0 mg./24 hrs. due to adrenal adenoma, against 7.0 - 29.0 mg./24 hrs. in a normal group.

Mlynaryk et al (1962) obtained significantly higher means in obese subjects than in normals (23.9 ± 6.0 mg./24 hrs. against 16.1 ± 6.5 mg./24 hrs.) and Schteingart et al (1963) also obtained higher means in an obese group (24.46 ± 2.92 mg./24 hrs. against 15.07 ± 1.3 mg./24 hrs, mean and S.E.). The difference between these two means was significant. In a third group of patients with Cushing's syndrome, these workers obtained a mean of 52.74 ± 5.0 (S.E.) mg./24 hrs. which was significantly higher than the mean of either the normal or obese group. None of their cases with Cushing's syndrome overlapped with the control group but two cases overlapped with the obese group and 15 of the 24 obese subjects showed higher-than-normal values. Gogate and Prunty (1963), however, obtained a range of 10.4 - 37.5 mg./24 hrs. for an obese group against 7.0 - 29.0 mg./24 hrs. for normal subjects with only 2 obese cases higher than normal.

(c) Excretion of 17-KS

Marked overlapping occurred among the range of values in the four groups; the levels reached by 6 normals and 6 obese subjects overlap with those reached by 4 patients with Cushing's syndrome. Again the mean in the group with Cushing's syndrome is significantly higher than either that of the normal group or the obese group. The difference between the means of the normal and obese groups is not significant.

The range obtained in normal subjects in this study compares well with that reported by James et al (1962), namely 4.4 - 14.2 mg./24 hrs. (8.7 ± 0.83; mean and S.E.). The sensitivity of estimations of 17-KS as a measure of/

of adrenocortical activity especially in diagnosis of Cushing's syndrome, has been doubted by many workers. Levell et al (1957) observed an excretion of 4.0 - 40.0 mg./day in 7 cases of Cushing's syndrome; raised value was noticed only in one case. Cope and Black (1959 a) reported insignificant rise in Cushing's syndrome (1.4 times the normal) and very poor relationship between this method and cortisol production rate estimation; values of 25 mg. for the former and 10 mg. for the latter or 30 mg. for the former and 225 mg. for the latter were associated. These workers stated that the relationship between CPR and urinary 17-KGS excretion was more encouraging. Prunty (1961) also reported that the upper limit of normal for this method was exceeded in 6 of 12 patients with Cushing's syndrome and adrenal hyperplasia.

Hoet et al (1961) reported a mean for normal subjects of 13.0 ± 3.9 mg./24 hrs. and 10.0 ± 3.6 mg./24 hrs. for males and females respectively and 10.4 ± 3.7 mg./24 hrs. for obese subjects and a range of 2.3 - 30.7 mg./24 hrs. for cases of Cushing's syndrome. Brooks et al (1963) reported a range of 1.0 - 38.0 mg./24 hrs. in adrenal hyperplasia, 9.0 - 20.0 mg./24 hrs. in adrenal adenoma against 4.0 - 17.0 mg./24 hrs. and 6.0 - 26.0 mg./24 hrs. in normal females and males respectively. Values were raised only in 9 of their 25 cases of Cushing's syndrome. Gogate and Prunty (1963) obtained similar values for obese and normals, namely 8.0 - 23.0 mg./24 hrs. for obese subjects, against 4.0 - 17.0 mg./24 hrs. and 6.0 - 26.0 mg./24 hrs. for normal females and males respectively. Only in one obese case was the upper limit of normal exceeded. Baird (1963) reported a mean of 14.5 ± 1.9 (S.E.) mg./24 hrs. in an obese group in some of whom Cushing's syndrome was suspected against 13.7 ± 0.76 mg./24 hrs. in normal subjects. Many of the former group were above normal, but the difference/

difference was not significant.

Dyrenfurth et al (1960), however, reported a high mean in Cushing's syndrome, namely 28.6 mg./24 hrs. and 20.6 mg./24 hrs. for males and females against 16.4 mg./24 hrs. and 10.9 mg./24 hrs. in normal subjects, and Borrell (1961) reported a range of 7.3 - 21.5 mg./24 hrs. in Cushing's syndrome against a normal mean of 6.14 mg./24 hrs. Similarly Rosner et al (1963) reported elevated values in 3 out of 5 obese subjects, but found that values in all cases with Cushing's syndrome were higher than normal.

(d) Excretion of total 17-KGS

Overlapping, more marked here than in the urine method of cortisol production rate is also present among the range of values of the four groups; the levels reached by 5 normal and 5 obese subjects overlap with those reached by 5 cases with Cushing's syndrome, but once more the mean in the group of Cushing's syndrome is significantly higher than either that of the normal or the obese group. The difference between the means of the normal and obese groups is not significant.

Other workers, also using the method of Few (1961), reported similar ranges and means for the normal subjects. Thus Michelakis (1962) reported a range of 10.1 - 20.5 and 6.3 - 17.4 mg./24 hrs., Rutherford and Nelson (1963) 9.0 - 17.0 mg./24 hrs. and 7.0 - 12.0 mg./24 hrs. and James and Caie (1964) 7.6 - 19.5 mg./24 hrs. and 4.9 - 15.1 mg./24 hrs. for males and females respectively.

Investigators using the 17-KGS method to compare normal, obese and subjects with Cushing's syndrome reported the following ranges and means. Levell et al (1957)/

(1957): 6 - 34 mg./24 hrs. in 7 cases of Cushing's syndrome, 4 of these showed elevated values, Szenas and Pattee (1959): 5.36 ± 0.38 (S.E.) mg./24 hrs. in 16 obese subjects and 3.49 ± 0.4 mg./24 hrs. in normals, with considerable overlap in the individual results, Dyrenfurth et al (1960): 5.45 mg./24 hrs. and 3.83 mg./24 hrs. in normal against 14.4 mg./24 hrs. and 11.2 mg./24 hrs. for males and females in a group of patients with Cushing's syndrome, Ross (1960): 10 - 46 mg./24 hrs. in Cushing's syndrome against 5.0 - 20.0 mg./24 hrs. in normals. Few (1961) estimating the 11-oxy- 17-KGS obtained the range of 7.1 - 13.5 mg./24 hrs. in normals, a mean of 32.4 mg./24 hrs. in Cushing's syndrome and 16.4 mg./24 hrs. in 'Cushing-type of obesity'. Hoet et al (1961) reported a mean of 11.1 ± 3.1 (S.E.) mg./24 hrs. and 9.8 ± 2.3 mg./24 hrs. for normal males and females against 12.0 ± 1.3 mg./24 hrs. for obese subjects and 13.5 - 49.5 mg./24 hrs. in Cushing's syndrome. Simkin and Arce (1962) obtained the range of 5.0 - 18.0 mg./24 hrs. (mean 11.0) in females and 8.0 - 25.0 mg./24 hrs. (mean 16.1) in males of a normal group against a mean of 15.8 ± 0.67 mg./24 hrs. in an obese group of whom those with coloured striae had a mean of 20.4 ± 1.62 mg./24 hrs. These last named workers noticed that 78% of the obese females with coloured striae had their 17-KGS exceed the upper limit of normal. Brooks et al (1963) found a range of 8.0 - 28.0 mg./24 hrs. in cases of adrenal hyperplasia and 10.0 - 21.0 mg./24 hrs. in adrenal adenoma against 5.0 - 18.0 mg./24 hrs. and 6.0 - 22.0 mg./24 hrs. for normal males and females respectively; the level was increased above normal in 13 of the 25 cases of Cushing's syndrome. Baird (1963) obtained a mean of 19.6 ± 1.89 (S.E.) mg./24 hrs. in an obese group, in some of whom Cushing's syndrome/

syndrome was strongly suspected, against a mean of 15.6 ± 0.71 mg./24 hrs. in normal subjects. The majority of levels in this obese group were beyond the normal range. Schteingart et al (1963) also obtained a mean of 8.97 ± 1.67 mg./24 hrs. (S.D.) in an obese group against 5.37 ± 0.55 mg./24 hrs. in normals (the difference was significant) and 13.97 ± 2.3 mg./24 hrs. in a group of Cushing's syndrome. The mean in the last group was significantly higher than in normal or obese groups, but considerable overlapping occurred among the ranges of the three groups.

Gogate and Prunty (1963), however, reported a range of 8.0 - 16.0 mg./24 hrs. in an obese group against 5.0 - 18.0 mg./24 hrs. and 6.0 - 22.0 mg./24 hrs. in normal females and males respectively; all the obese subjects were within the normal range.

Commenting on the sensitivity of the 17-KGS estimations, Cope and Black (1959 a) reported poor correlation between this estimation and estimations of cortisol production rate; in low, normal or moderately raised adrenal activity, the relationship was poor. Prunty (1961) stated that cortisol production rate estimations are more sensitive than 17-KGS estimations; recovery of cortisol as 17-KGS was between 12 - 46% and that levels in Cushing's syndrome were slightly elevated or within the upper limit of normal in 6 out of 18 cases. Grant (1963) also believes that the correlation between these two measurements is not always very good.

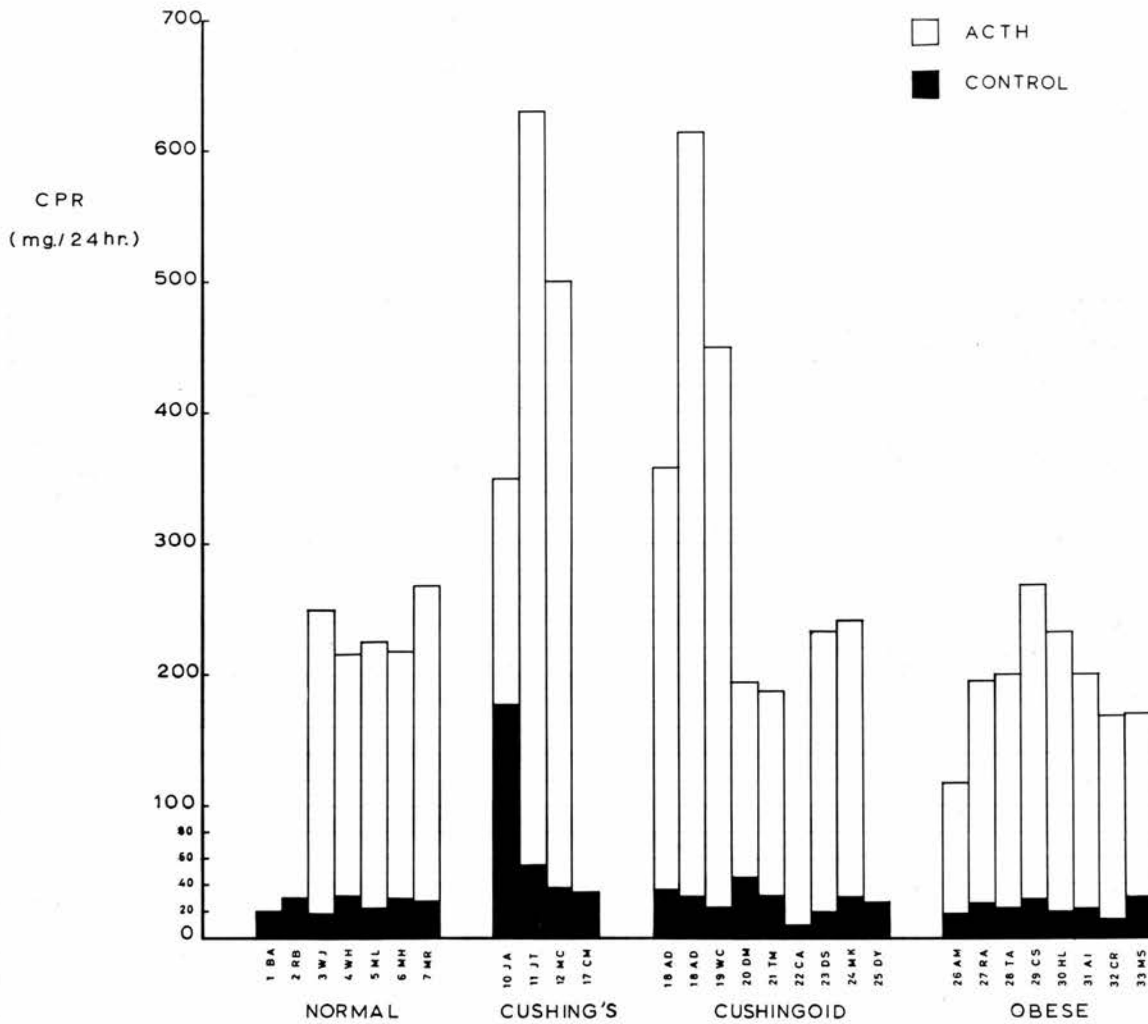

FIGURE 18: CORTISOL PRODUCTION RATE (BLOOD METHOD)

CONTROL ESTIMATIONS AND ESTIMATIONS AFTER ACTH

3. Cortisol production rate (blood method), estimations after ACTH stimulation

Table 11 shows the results of estimation of cortisol production rate by the blood method in the four groups after ACTH stimulation (see also figure 18).

(a) The miscible pool

The range of the normal subjects is clearly separated from that of the patients with Cushing's syndrome at the range of 12.1 - 13.8 mg. as all normal subjects have values below 12.1 mg. whereas all patients with Cushing's syndrome have values above 13.8 mg. There are overlaps amongst the other ranges. The increase of the mean for the group of patients with Cushing's syndrome above the means for either the normal or obese group is significant. No significant difference exists between the mean for the normal and obese groups nor between the 'Cushingoid' and the other groups. In each group, however, the rise of the mean above the corresponding control mean is highly significant (see tables 9, 28 and 29).

(b) The biological half clearance time and the rate of turnover

There are marked overlaps among the ranges of the four groups. In estimations of the rate of turnover, only the mean of the 'Cushingoid' group is significantly lower than that of either the normal group or the group of Cushing's syndrome. However, the difference between the mean of each group and its corresponding control mean is significant in the group of Cushing's syndrome and the obese group and highly significant in the normal and 'Cushingoid' group denoting increased rate of turnover after ACTH stimulation.

(c) Cortisol production rate

After ACTH, the normal and simple obese subjects are widely separated from/

Table (11)

Cortisol production rate (blood method)

Estimations after ACTH stimulation

Case	Age	Sex	Miscible pool	$t_{\frac{1}{2}}$	Rate of turnover	CPR	CPR: increase over control	
	(yrs)		(mg.)	(min.)	(pool/hr)	(mg./24hr)	(mg)	(%)
1. Normal group								
3. W.J.	35	M	12.1	48	0.86	249	231	1326
4. W.H.	55	M	7.5	33	1.20	216	185	592
5. M.L.	42	F	9.4	39	1.00	225	203	934
6. M.H.	35	F	10.1	45	0.90	218	189	658
7. M.R.	23	F	10.5	39	1.06	268	241	899
Range	23-55		7.5-12.1	33-48	0.86-1.20	216-268	185-241	592-1326
Mean	38		9.9	40.8	1.00	235	210	882
S.D.			1.6		0.14	22	24	288
S.E.			0.7		0.06	10	11	128
2. Cushing's group								
a. operated upon								
10. J.A.	55	M	13.8	39	1.10	350	172	97
11. J.T.	17	F	24.6	39	1.07	631	576	1053
12. M.C.	36	F	18.1	36	1.16	501	463	1223
Range	17-55		13.8-24.6	36-39	1.07-1.16	350-631	172-576	97-1223
Mean	36		18.8	38.0	1.11	494	404	791
S.D.			5.3		0.047	140	20	606
S.E.			3.0		0.027	81	12	350

Table (11) contd

Case	Age (yrs)	Sex	Miscible pool (mg.)	$t\frac{1}{2}$ (min.)	Rate of turnover (pool/hr)	CPR (mg./24hr)	CPR: increase over control (mg)	CPR: increase over control (%)
3. 'Cushingoid' group								
18. A.D.	19	F	24.5	68	0.61	358	321	869
19. W.C.	27	M	23.2	51	0.81	450	427	1858
20. D.M.	21	M	11.3	57	0.72	194	148	327
21. T.M.	18	M	15.0	80	0.52	187	156	500
23. D.S.	34	F	9.8	42	0.99	233	214	1147
24. M.K.	15	F	11.7	48	0.86	241	211	699
Range	15-34		9.8-24.5	42-80	0.52-0.99	187-450	148-427	327-1858
Mean	22.3		15.9	57.6	0.75	277	246	900
S.D.			6.3		0.171	104	107	596
S.E.			2.6		0.071	42	44	243
4. Obese group								
26. A.M.	34	M	4.2	36	1.16	117	99	553
27. R.A.	12	M	10.0	51	0.81	195	169	653
28. T.A.	17	M	8.4	42	0.99	199	177	801
29. C.S.	14	F	11.3	42	0.99	268	239	833
30. H.L.	31	F	15.9	67	0.61	232	213	1144
31. A.I.	24	F	6.6	33	1.26	199	178	830
32. C.R.	20	F	5.0	30	1.40	168	155	1172
33. M.S.	14	F	9.5	57	0.74	169	138	454
Range	12-34		4.2-15.9	30-67	0.61-1.40	117-268	99-239	454-1172
Mean	20.8		8.8	44.8	1.00	193	171	805
S.D.			3.7		0.27	45	43	256
S.E.			1.3		0.095	15	15	90

from the patients with Cushing's syndrome. The increase in the mean of the group with Cushing's syndrome above the mean of either the normal or of the obese group is highly significant. It is also significantly greater than that of the 'Cushingoid' group. Differences between the means of the normal and obese groups are not significant. However, the rise in the mean in each group, after ACTH, over its corresponding control mean (see table 9) is highly significant.

Expressing the results of cortisol production rate estimation as "increase above the control value" in mg., results again in overlap, but still the difference between the mean of the group with Cushing's syndrome and that of either the normal or obese groups is highly significant. Differences between other means are not significant. On the other hand, expressing the results of cortisol production rate estimation as "percent increase above the control value", results in marked overlapping, and the differences between the means of any of the four groups is not significant. Thus, it seems best to record results of CPR after ACTH stimulation simply as the absolute value reached after stimulation and not as the increase over the control value.

Comparison of these results with those obtained by other workers cannot be made, because this dosage of ACTH has been used for the first time in the present study. DeMoor et al (1961 a), however, stated that the biological half clearance time was prolonged after ACTH (294 minutes against 163 in the control estimations) which is not in agreement with the present finding of significant shortening of this period. Peterson and Wyngaarden (1956) and Hoet et al (1961), on the other hand, found that the rate of turnover did not/

not significantly change from the control value. The latter workers found no difference between the miscible pools of normal and obese subjects (14.7 ± 5.6 mg. against 13.4 ± 4.7 mg.) on the first day of ACTH stimulation (intravenous infusion of 25 I.U. over 8 hrs.) but a much larger pool was noticed in 3 cases of Cushing's syndrome (41.2 - 59.5 mg.). On repeating the test on the second day the values for the three groups were 19.7 ± 5.3 mg., 24.1 ± 4.9 mg. and 49.2 - 59.0 mg. respectively. Accordingly the production rates as calculated for the three groups were 7.56 ± 2.54 mg./hr. (≡ 181.44 ± 60.96 mg./24 hrs.), 8.4 ± 3.0 mg./hr. (≡ 201.6 ± 72.0 mg./24 hrs.) and 26.7 - 51.5 mg./hr. (≡ 640 - 1238 mg./24 hrs.) for the first day and 12.2 ± 2.5 mg./hr. (≡ 292.8 ± 60.0 mg./24 hrs.), 14.1 ± 3.5 mg./hr. (≡ 338.4 ± 84.0 mg./24 hrs.) and 41.9 - 50.1 mg./hr. (≡ 1005 - 1202 mg. /24 hrs.) for the second day. Although values of the second day of ACTH stimulation are higher than those obtained in the present study the normal and obese subjects show similar means while cases of Cushing's syndrome show more hyper-responsiveness. Nugent et al (1963) had stated that 110 µg./Kg./hr. is the rate at which normal subjects produce cortisol in response to maximal stimulation with ACTH for 6 hours. This corresponds to about 160 mg./day for a 60-Kg. subject. Values obtained in this study were higher, which indicates that the dosage of ACTH used by Nugent and his co-workers were inadequate to produce maximal stimulation of the adrenal cortices.

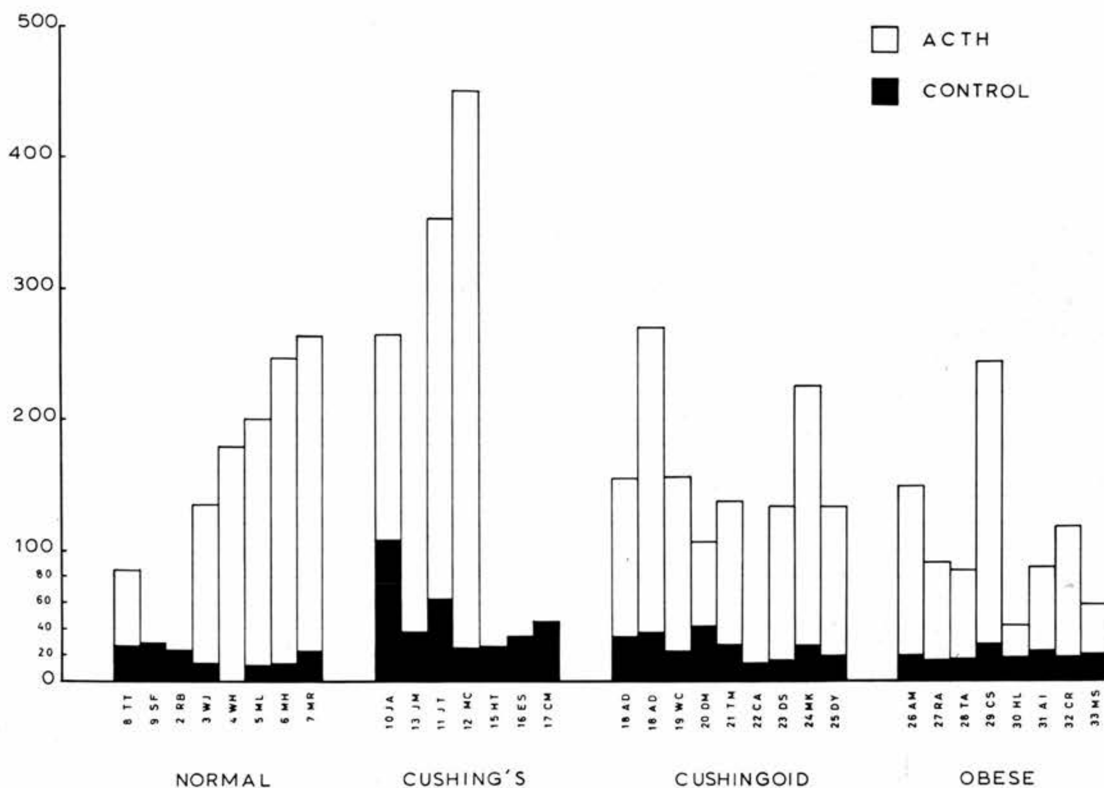

FIGURE 19: CORTISOL PRODUCTION RATE (URINE METHOD)

CONTROL ESTIMATIONS AND ESTIMATIONS AFTER ACTH

4. <u>Cortisol production rate (urine method), estimations after ACTH stimulation</u>

Table 12 shows the results of estimation of cortisol production rate by the urine method after ACTH stimulation (see also figure 19). Whenever the blood method was performed, the urine method was carried out on the same day for the sake of comparison.

(a) <u>Percent of radioactive cortisol dose excreted in urine in the first 24 hrs.</u>

Again, of all the 27 subjects studied, 21 excreted more than 70% of the radioactive dose, 20 more than 75% and 17 more than 80%. No significant differences existed between any two means of all four groups nor between the mean of any group, after ACTH stimulation, and its corresponding control mean.

Cope and Black (1958 b) reported excretion of 58 - 89.5% (mean 76.4) of a radioactive cortisol dose in the first 24 hrs. in normal subjects which is in agreement with the present findings.

(b) <u>Cortisol production rate</u>

The normal and obese subjects were again clearly but narrowly separated from the patients with Cushing's syndrome; the upper limit of the normal group did not overlap with the lower limit of the group of Cushing's syndrome. The mean in the group of Cushing's syndrome is significantly higher than that of either the normal and 'Cushingoid' group and the difference between the means of the group with Cushing's syndrome and the obese group is highly significant. The difference between the means of the normal and obese groups was not significant. Again, the increase in the mean of each group, after ACTH stimulation, over its corresponding control mean is highly significant.

Expressing the results of cortisol production rate as "increase above the control value" in mg., results in overlapping among the ranges. The difference/

Table (12)

Cortisol production rate (urine method)

Estimations after ACTH stimulation

Case	Age	Sex	Dose excretion first 24 hr.	S.A. THE	CPR	CPR: increase over control	
	(yrs)		(%)	(cpm/µg.)	(mg./24hr)	(mg)	(%)
1. Normal group							
3. W.J.*	35	M	77.7	15.6	134	120	893
4. W.H.*	55	M	81.3	12.4	178	-	-
8. T.T.	72	M	80.0	4.9	85	58	219
5. M.L.*	42	F	80.4	10.7	199	187	1617
6. M.H.*	35	F	82.1	9.0	246	233	1806
7. M.R.*	23	F	76.7	7.6	262	240	1073
Range	23-72		76.7-82.1		85-262	58-240	219-1806
Mean	43.6		79.7		184	168	1122
S.D.			2.0		67	202	902
S.E.			0.8		27	90	403
2. Cushing's group							
a. Operated upon							
10. J.A.	55	M	52.3	1.8	263	156	146
11. J.T.	17	F	87.4	3.1	355	293	470
12. M.C.	36	F	80.2	2.2	450	425	1729
Range	17-55		52.3-87.4		263-450	156-425	146-1729
Mean	36		73.3		356	291	782
S.D.			18.5		93	134	835
S.E.			10.7		53	77	482

Table (12) contd

Case	Age	Sex	Dose excretion first 24 hr.	S.A. THE	CPR	CPR: increase over control	
	(yrs)		(%)	(cpm/μg.)	(mg./24hr)	(mg)	(%)
			3.	'Cushingoid' group			
18. A.D.	19	F	84.8	5.3	154	121	365
19. W.C.	27	M	83.6	5.0	155	133	601
20. D.M.*	21	M	86.3	14.8	106	65	158
21. T.M.	18	M	90.9	9.3	137	110	411
23. D.S.*	34	F	83.0	14.8	132	117	784
24. M.K.	15	F	76.7	8.3	225	198	722
25. D.Y.*	32	F	82.1	25.3	132	113	609
Range	15-34		76.7-90.9		106-225	65-198	158-784
Mean	23.7		83.9		149	122	521
S.D.			4.3		37	39	210
S.E.			1.6		14	14	79
			4.	Obese group			
26. A.M.	34	M	69.8	5.0	148	129	670
27. R.A.	12	M	81.0	8.5	89	73	478
28. T.A.*	17	M	88.1	27.8	83	67	429
29. C.S.	14	F	86.7	3.9	242	214	776
30. H.L.	31	F	74.9	17.0	42	25	148
31. A.I.*	24	F	89.8	19.5	86	63	268
32. C.R.	20	F	45.5	4.1	118	100	564
33. M.S.	14	F	91.2	16.6	57	37	195
Range	12-34		45.5-91.2		42-242	25-214	148-776
Mean	20.8		78.3		108	89	443
S.D.			13.2		63	60	224
S.E.			4.6		22	21	79

* Patients received ACTH twice daily for 3 days. All other patients received ACTH twice daily for 2 days and once on the morning of the third day. In both cases estimations were carried out on the third day.

Table (12 a)

Comparison of CPR estimations by blood and urine method

Case	CPR control mg./24 hrs.		CPR ACTH mg./24 hrs.	
	Blood method	Urine method	Blood method	Urine method
1. Normal group				
2. R.B.	30.0	23.7	-	-
3. W.J.	17.5	13.5	250	134
4. W.H.	-	-	216	179
5. M.L.	21.8	11.6	226	199
6. M.H.	28.8	12.9	218	246
7. M.R.	26.9	22.4	269	263
2. Cushing's group				
10. J.A.	177.6	107.0	350	264
11. J.T.	54.7	62.2	631	355
12. M.C.	37.9	24.6	501	450
17. C.M.	34.1	44.7	-	-
3. 'Cushingoid' group				
18. A.D.	37.0	33.2	359	155
repeated	31.0	36.1	614	268
19. W.C.	23.0	22.1	451	155
20. D.M.	45.6	41.2	195	106
21. T.M.	31.2	26.8	187	137
22. C.A.	8.6	13.4	-	-
23. D.S.	18.7	15.0	233	133
24. M.K.	30.2	27.4	241	226
25. D.Y.	26.4	18.7	-	-
4. Obese group				
26. A.M.	18.0	19.2	118	148
27. R.A.	25.9	15.5	195	90
28. T.A.	22.1	15.8	199	84
29. C.S.	28.8	27.7	269	243
30. H.L.	18.7	17.0	233	42
31. A.I.	21.4	22.5	199	87
32. C.R.	13.2	17.8	168	118
33. M.S.	30.5	19.6	169	58

difference between the mean of the group of Cushing's syndrome and the obese group is highly significant, whereas the difference between any other two means is not significant. Again expressing the results of cortisol production rate estimation as "percent increase above the control value", results in marked overlapping and the mean of the normal group is significantly increased over that of both the 'Cushingoid' and the obese group. Thus it seems best to describe the results of ACTH stimulation in terms of absolute value reached after ACTH stimulation.

Comparison between these figures and those of other workers is impossible because of the unique dosage of ACTH used in the present study.

Comparison of the cortisol production rates obtained by blood and urine methods in individuals reveals a discrepancy; the values given by the blood method were on the whole higher than those given by the urine method (see table 12, a). Administration of ACTH in 6 doses instead of 5 did not remove the difference; the results of the urine method remained well below those of the blood method. This suggests that a plateau of increased cortisol secretion must have been attained after 5 doses of ACTH.

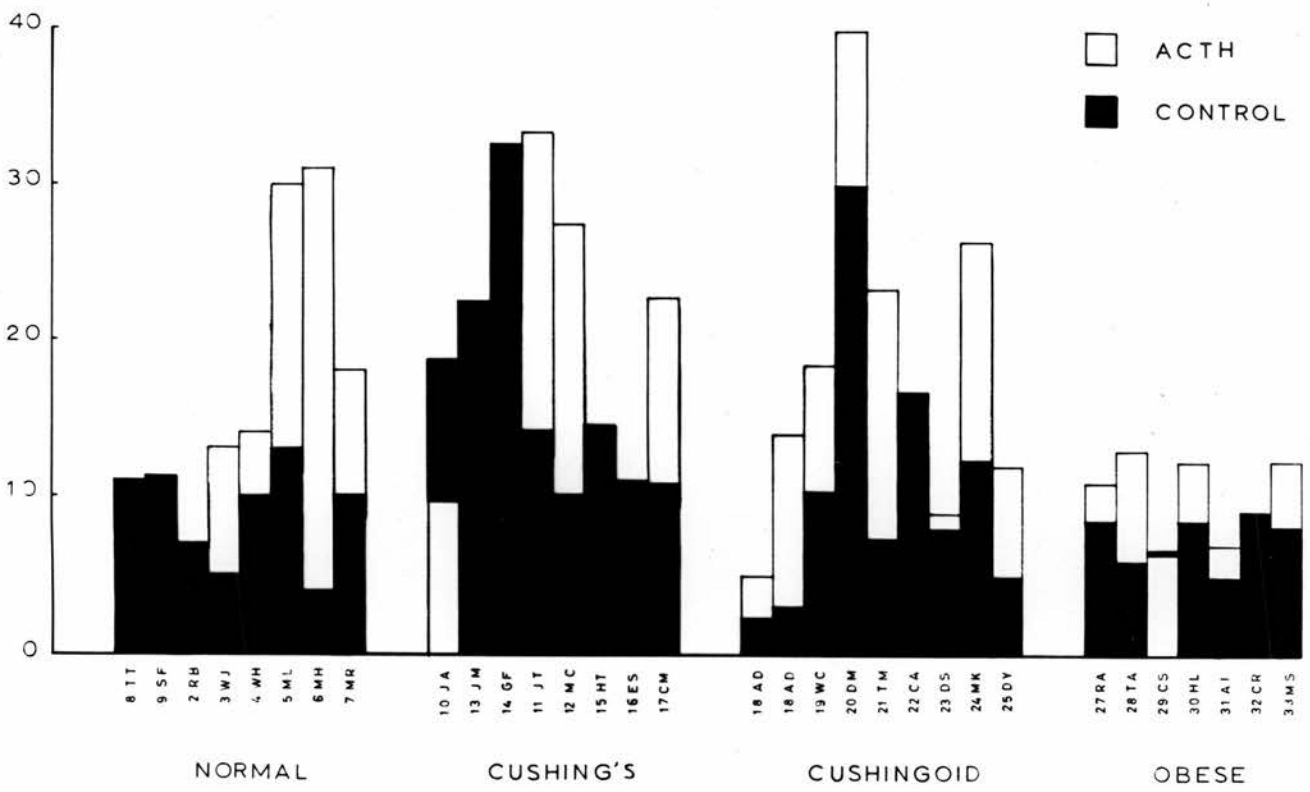

FIGURE 20: URINARY 17-KETOSTEROID EXCRETION

CONTROL ESTIMATIONS AND ESTIMATIONS AFTER ACTH

5. Estimations of urinary 17-KS and total 17-KGS after ACTH stimulation

Table 13 shows the results of estimation of urinary 17-ketosteroids and total 17-ketogenic steroids after ACTH stimulation (see also figures 20 and 21). Whenever cortisol production rate was estimated, these tests were carried out on the same day for the sake of comparison.

(a) Excretion of 17-KS

Overlapping exists except between the normal and obese group; values in 5 normal subjects and in 4 obese patients overlap with those of 3 patients with Cushing's syndrome. The increase in the mean of the normal group over that in the obese group is highly significant and the increase in the group of Cushing's syndrome over the obese group is significant. No significant difference exists between the means of the normal group and the group of Cushing's syndrome nor between that of the 'Cushingoid' and those of the other groups. Considering the rise in the mean of each group, after ACTH stimulation, above the corresponding control mean, a highly significant difference exists for the normal group, a significant difference exists for the obese group but no significant difference exists for either the group of Cushing's syndrome or the 'Cushingoid' group. It was observed in one patient with Cushing's syndrome and in one obese subject that the value of 17-KS excretion after ACTH was lower than the control value which is evidence that this method does not reflect the actual state of adrenocortical function.

Expressing the results of 17-KS excretion as "increase above the control value" in mg., results in marked overlap and only the differences between the means of both the normal and 'Cushingoid' group and that of the obese group is/

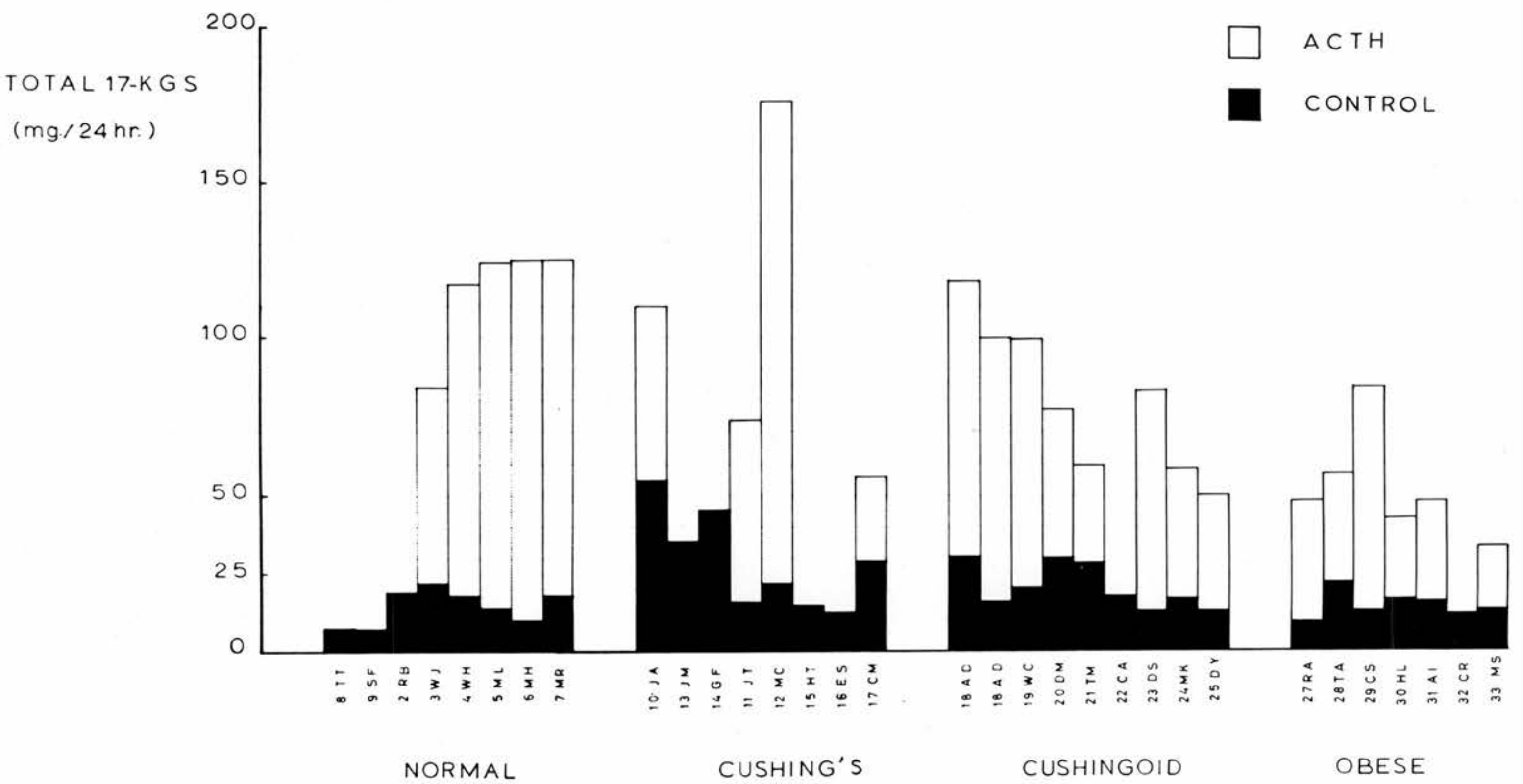

FIGURE 21: URINARY TOTAL 17-KETOGENIC STEROID EXCRETION

CONTROL ESTIMATIONS AND ESTIMATIONS AFTER ACTH

Table (13)

Urinary 17-KS and total 17-KGS excretion

Estimations after ACTH stimulation

Case	Age	Sex	17-KS	17-KS: increase over control		Total 17-KGS	Total 17-KGS increase over control	
	(yrs)		(mg./24hr.)	(mg)	(%)	(mg/24hr.)	(mg)	(%)
1. Normal group								
3. W.J.*	35	M	13.0	8.0	160	84	62	281
4. W.H.*	55	M	14.0	4.0	40	117	99	550
5. M.L.*	42	F	30.0	17.0	130	124	110	785
6. M.H.*	35	F	31.0	27.0	675	125	115	1150
7. M.R.*	23	F	18.0	8.0	80	125	107	594
Range	23-55		13.0-31.0	4.0-27.0	40-675	84-125	62-115	281-1150
Mean	38		21.2	12.8	217	115	98	672
S.D.			0.8	9.2	26	17	21	321
S.E.			0.3	4.1	11	7	9	143
2. Cushing's group								
a. Operated upon								
10. J.A.	55	M	9.7	-9.0	-48	109	54	99
11. J.T.	17	F	33.4	19.2	135	73	57	365
12. M.C.	36	F	27.5	17.3	169	176	154	708
b. Not operated upon								
17. C.M.	18	F	22.7	11.9	110	56	27	93
Range	17-55		9.7-33.4	-9.0-19.2	-48-169	56-176	27-154	93-708
Mean	31.5		23.3	9.8	91	103	73	316
S.D.			10.3	10.6	87	53	17	290
S.E.			5.1	5.3	43	26	8	145

Table (13) contd

Case	Age	Sex	17-KS	17-KS: increase over control		Total 17-KGS	Total 17-KGS: increase over control	
	(yrs)		(mg./24hr)	(mg)	(%)	(mg./24hr)	(mg)	(%)
3. 'Cushingoid' group								
18. A.D.	19	F	5.0	2.7	117	118	87	286
19. W.C.	27	M	18.4	8.1	78	99	78	382
20. D.M.*	21	M	40.0	10.0	33	77	47	156
21. T.M.	18	M	23.3	15.9	214	59	30	105
23. D.S.*	34	F	9.0	1.0	12	83	70	538
24. M.K.*	15	F	26.4	14.0	112	58	41	246
25. D.Y.*	32	F	12.0	7.0	140	50	37	284
Range	15-34		5.0-40.0	1.0-15.9	12-214	50-118	30-87	105-538
Mean	23.7		19.1	8.4	101	78	56	285
S.D.			11.9	5.0	68	24	22	143
S.E.			4.5	1.9	25	9	8	54
4. Obese group								
27. R.A.	12	M	10.9	2.3	26	48	38	409
28. T.A.*	17	M	13.0	7.0	116	57	35	159
29. C.S.	14	F	6.6	-0.1	-1.4	84	71	546
30. H.L.	31	F	12.3	3.7	43	42	26	161
31. A.I.*	24	F	7.0	2.0	40	48	32	200
33. M.S.	14	F	12.3	4.1	50	33	20	157
Range	12-31		6.6-13.0	-0.1-7.0	-1.4-116	33-84	20-71	157-546
Mean	18.6		10.4	3.2	45	52	37	272
S.D.			2.8	2.3	39	17	17	165
S.E.			1.1	0.9	15	7	7	67

* Patients received ACTH twice daily for 3 days. All other patients received ACTH twice daily for 2 days and once on the morning of the third day. In both cases estimations were carried out on the third day.

is significant. Again, expressing the results of 17-KS excretion as "percent increase above the control value", results in a more marked overlap and the difference between any two means of the four groups is not significant.

(b) Total 17-KGS excretion

Overlapping is present except between the ranges of normal and obese groups. Values in 5 normal and 2 obese subjects overlapped with those of 3 patients with Cushing's syndrome. The increase in the mean of the normal group over that of the obese one is highly significant, the increase in the mean of the normal over that of the 'Cushingoid' group and in the mean of the patients with Cushing's syndrome over that of the obese group is significant. No significant difference exists between the means of the normal subjects and the patients with Cushing's syndrome. The increase in the mean of each group, after ACTH, over its corresponding control mean is highly significant.

Expressing the results of total 17-KGS excretion as "increase above the control value" in mg. shows overlapping among all means, but the difference between the mean of the normal group and those of both the 'Cushingoid' and obese group is highly significant. Expressing these results as "percent increase above the control value", produces marked overlapping among the mean of all four groups and only the difference between the mean of the normal group and those of both the 'Cushingoid' and obese groups is significant. Expressing the results as their absolute values, therefore, provides the most useful information. This agrees with the finding of Beck et al (1962) who found significant increase of 17-KGS excretion, after ACTH stimulation, of the mean/

mean between a normal group and a group of Cushing's syndrome, but when the results were expressed as increase in mg. over control the means overlapped and when expressed as percent increase over control value the normal mean was nearly double that of the Cushing's.

It has been known for a long time that the relationship between CPR and urinary steroid excretion gets even poorer at higher secretion states. Cope and Black (1959 a) reported that at high rates of adrenocortical activity, the excretion of 17-KGS is approximately half the CPR. James and Caie (1964), employing the same method used in this study (Few, 1961), reported that 17-KGS values were 78 - 92% of CPR up to 35 mg./day but only 26 - 55% of it above that. They suggested that the decreased conversion of cortisol to 17-KGS at high secretion rates might be due to increased formation of metabolites which were not determined as 17-KGS, i.e. probably polar metabolites.

No detailed comparison can be made with figures obtained by other workers, comparing normal subjects, obese subjects and patients with Cushing's syndrome, because of the different dosage of ACTH. Poisnick and DiRaimondo (1956), however, noticed a 30% increase in the mean of an obese group above that of a normal group, but the ratio of the stimulated to the basal levels were the same in both groups.

Birke et al (1960) reported that in cases of Cushing's syndrome due to hyperplasia of the adrenal cortex, the upper limit of normal was exceeded, but they observed only a weak response in adenoma and no response in adrenal carcinoma. They suggested that the increase in patients with hyperplasia was due/

due to the presence of an increased functioning mass of the adrenal cortex. These workers found that 17-KS estimations were less sensitive than 17-KGS; two patients with adrenal hyperplasia and the patients with adrenal adenoma showed normal resting excretion and normal response to ACTH stimulation. They concluded that the usefulness of 17-KS or their variation from day-to-day in patients with Cushing's syndrome due to adrenocortical hyperplasia was limited.

Dyrenfurth et al (1960) obtained significantly higher results for 17-KGS excretion in Cushing's syndrome than in normals. 17-KS determinations were again less sensitive. Hoet et al (1961) obtained identical means in normal and obese subjects after ACTH stimulation but much higher levels in Cushing's syndrome. Rosner et al (1963) also reported a normal response to stimulation in obese subjects and Baird (1963) noticed that the level of 17-KGS after ACTH, in an obese group, rose but that the rise was slight. Gogate and Prunty (1963) also observed normal adrenal response in an obese group after ACTH stimulation, as measured by both 17-KGS and 17-KS estimations; the latter test was as usual less sensitive than the former.

The most brisk response of 17-KGS to ACTH stimulation in the present study was obtained in case 12 (M.C.) who later on at operation proved to have nodular hyperplasia with adenomata. Nabarro et al (1958) also observed unexpected responses of supposedly autonomous tumours, and Prunty (1961) stated that one of his patients with adenoma responded well to ACTH.

6. Cortisol production rate (urine method) and urinary steroid excretion after suppression with dexamethasone

Table 14 demonstrates the results obtained after dexamethasone suppression. None of the subjects of the normal group was studied by this parameter. In each case included in table 14, an attempt was made to estimate the cortisol production rate by the blood method, but suppression was so marked that the fluorometric readings of the eluates of the plasma chromatograms were (except in one case, J.A.) the same as that of the paper blank. Accordingly, it was not possible to estimate the specific activities of the blood samples from which the production rate could be calculated. Only in one patient in the group of Cushing's syndrome (case 10. J.A., after unilateral adrenalectomy) suppression was not so marked and estimation of a production rate of 33.6 mg./24 hrs. was made possible from a miscible pool of 1.4 mg. and a rate of turnover of 0.99 pool/hr. (biological half clearance time 42 minutes). This represents a drop of about 65% and 32% of the pre-operative value for the miscible pool and CPR respectively.

(a) Percent of the radioactive cortisol dose excreted in the first 24 hrs

Again, of all 16 subjects studied, 15 excreted more than 70% of the radioactive dose, 13 more than 75% and 11 more than 80%. No significant difference exists between any two of those means and no significant difference exists between the mean of any of these groups, after dexamethasone, and its corresponding control mean.

(b) Cortisol production rate estimation by the urine method

Marked suppression occurred in all cases except case 10. (J.A., see also table 22). The difference between any two means is not significant. However, the/

Table (14)

Cortisol production (urine method) and urinary steroid excretion

Estimations after suppression with dexamethasone

Case	Age (yrs)	Sex	Dose excretion first 24 hr (%)	S.A. THE (cpm/μg)	CPR (mg./24hr)	17-KS (mg./24hr)	Total 17-KGS (mg./24hr)
2. Cushing's group							
a. operated upon							
10. J.A.**	55	M	72.5	16.7	39.5	3.0	15.7
11. J.T.	17	F	81.9	284.5	3.1	4.0	5.2
12. M.C.	36	F	83.3	157.7	3.7	-	-
Range	17-55		72.5-83.8		3.1-39.5	3.0-4.0	5.2-15.7
Mean	36		79.4		15.4	3.5	10.5
S.D.			6.0		20.8	0.7	7.4
S.E.			3.4		12.0	0.4	5.2
3. 'Cushingoid' group							
18. A.D.	19	F	91.4	53.6	3.4	1.0	2.4
19. W.C.	27	M	91.5	430.0	2.0	4.7	6.7
20. D.M.	21	M	86.8	846.4	1.7	6.0	5.0
21. T.M.	18	M	81.3	232.6	3.7	-	-
23. D.S.*	34	F	81.6	2009.6	1.1	2.0	1.0
Range	18-34		81.3-91.5		1.1-3.7	1.0-6.0	1.0-6.7
Mean	24		86.5		2.4	3.4	3.8
S.D.			5.0		1.0	2.3	2.5
S.E.			2.2		0.4	1.1	1.1
4. Obese group							
26. A.M.	34	M	78.5	206.0	4.0	-	-
27. R.A.	12	M	79.7	626.5	1.2	2.8	1.1
28. T.A.	17	M	88.7	928.9	2.5	3.0	5.0
29. C.S.	14	F	72.0	306.9	2.5	7.2	4.3
30. H.L.	31	F	85.3	238.4	2.1	3.8	4.2
31. A.I.*	24	F	83.2	433.7	5.0	3.0	5.0
32. C.R.	20	F	48.6	66.0	7.8	3.7	2.5
33. M.S.	14	F	87.4	405.5	2.0	2.5	3.8
Range	12-34		48.6-88.7		1.2-7.8	2.5-7.2	1.1-5.0
Mean	20.8		77.9		3.4	3.7	3.7
S.D.			13.0		2.0	1.6	1.6
S.E.			4.6		0.7	0.6	0.6

* Dexamethasone was administered to these patients 4 times daily for 2 days and estimations carried out on the second day. To all the other patients dexamethasone was administered 4 times daily for 2 days and once on the morning of the third day and estimations carried out on the third day.

** Estimations carried out after unilateral adrenalectomy.

the drop in the mean from its corresponding control value was highly significant in the 'Cushingoid' and obese groups but not in the group with Cushing's syndrome (see table 29).

No attempts were made by other workers to estimate CPR after dexamethasone suppression.

(c) <u>17-KS excretion</u>

Marked suppression occurred in all cases and there was no difference between any two means after suppression. There is, however, a significant drop of the mean in the group of Cushing's syndrome from its corresponding control value, and a highly significant drop in the obese group but there was no significant drop in the 'Cushingoid' group.

(d) <u>Total 17-KGS excretion</u>

Marked suppression occurred in all cases except case 10, (J.A.). All the cases in the obese group, however, had values below 5.0 mg./24 hrs. whereas the two cases of Cushing's syndrome were above 5.2 mg./24 hrs. The difference between the means of the group with Cushing's syndrome and the obese group is significant, and the fall in the means of both the 'Cushingoid' and obese groups from their corresponding control means are highly significant, but the change is not significant in the patients with Cushing's syndrome.

It can be observed from examination of the individual cases in table 14 that, inspite of changing the dosage of dexamethasone to 8 doses instead of 9, and performing the test on the second day instead of the third, marked suppression still occurred, denoting that a plateau of diminished cortisol secretion must have been attained, starting from the second day of suppression with dexamethasone. The markedly low excretory values either on the second day (8 doses) or on the third/

third day (9 doses) denotes that this plateau of diminished cortisol secretion, and accordingly the effect of dexamethasone administration, could last to the end of the third day, even though the last dose was administered on the morning of that day.

Marked suppression of urinary 17-KGS in obese subjects with the smaller dosage (0.5 mg. every 6 hours for two days) have been reported by Simkin and Arce (1962), Slater et al (1962), Gogate and Prunty (1963), Rosner et al (1963) and Baird (1963) to values below 4 mg./day. Slater et al (1962) reported that cases of Cushing's syndrome always excreted values above 5 mg./day. Schteingart et al (1963) noticed that obese subjects were suppressed to 2.5 mg. or less, while patients with Cushing's syndrome excreted not less than 3.3 mg./24 hrs. Other results which support the usefulness of the dexamethasone test in diagnosing cases of Cushing's syndrome were obtained by Brooks et al (1963) who noticed that their cases of Cushing's syndrome, except one, failed to suppress in response to dexamethasone. Their 17-KS values were less sensitive; the changes in 17-KS excretion following dexamethasone suppression were much more variable, and the test was of less diagnostic value then 17-KGS determination.

Liddle (1960), who was the first to suggest the use of dexamethasone (0.5 mg./6 hrs. for 2 days then 2 mg./6 hrs. for another 2 days) as a useful tool in diagnosis of Cushing's syndrome, reported that normal subjects excreted 2.5 mg. or less/day, and patients with Cushing's syndrome due to hyperplasia 4.0 - 35.0 mg./day after dexamethasone. In another group of cases suspected of suffering from Cushing's syndrome, Liddle (1960) observed basal steroid levels which were higher than normal, and in this group excretion after dexamethasone/

dexamethasone was as low as 1.0 - 4.0 mg./day. He concluded that there were different resistances to suppression by dexamethasone in patients with bilateral adrenocortical hyperplasia, and that extreme cases might be encountered in which normal suppression with small doses or complete resistance to the large dose might be found. He also concluded that this test did not distinguish adrenal hyperplasia from tumour, but rather ACTH-dependent hyperadrenocorticism from autonomous adrenocortical hypersecretion, and accordingly that this test was not in itself sufficient evidence to justify a diagnosis of Cushing's syndrome; definite clinical features and elevated levels of cortisol metabolites should be present to justify the diagnosis. Liddle further stated that spontaneous diminution in adrenal secretory activity might coincide with administration of the suppressing agent, leading to a mistaken impression, and he recommended repetition of the test to make sure of its results. He noticed that 17-KS excretion did not fall as readily as the 17-KGS.

Dyrenfurth et al (1960), using 9α-FF, noticed some degree of suppression but there was no difference between patients with Cushing's syndrome and normal individuals, and Prunty (1961) observed that the suppression was very variable in a group of patients with Cushing's syndrome due to adrenal hyperplasia. He noticed also that a patient with adenoma responded well. This last observation supports the finding in the present study of marked suppression in a case of Cushing's syndrome with nodular hyperplasia and adenoma (Case 12, M.C.).

From the findings of the present study, the author believes that suppression of total 17-KGS with dexamethasone, at the best, separates normal and obese subjects on one hand from cases of Cushing's syndrome on the other, but that the separation may not be marked. In table 14, the lowest value for 17-KGS in

a/

a patient with Cushing's syndrome, following administration of dexamethasone, was 5.2 mg./24 hrs., and the highest value in an obese subject was 5.0 mg./24 hrs; the difference between these two being within the limits of experimental error. By contrast, the separation after ACTH as measured by CPR (blood method), is "magnified" to the wide range of 268 - 350 mg., thus rendering the latter technique much superior as a means of differential diagnosis.

7. Calculation of cortisol production rate from double compartment model

Comparison of the results of cortisol production rate estimations in individual cases shows a discrepancy between the blood method and the urine method in the control estimations and, more markedly, in the estimations after ACTH (see table 12 a). It was mentioned in the description of table 12 that the dosage of ACTH was not responsible for this discrepancy. It was considered desirable to investigate whether calculation of cortisol production rate by the blood method from a double compartment model could explain this discrepancy.

For estimations from the double compartment model, two extra blood samples were withdrawn in the interval 0 - 30 minutes, after injection of the radioactive cortisol; their time of withdrawal was recorded and their specific activities estimated as usual. The slope of the first part of the graph (S.A. against time) could be drawn by joining these two specific activities and that of the blood sample withdrawn at 30 minutes after injection of the dose into a straight line, and extrapolating it back to zero time specific activity on semilogarithmic graph. Cortisol production rate could then be calculated from the appropriate formula (see Introduction, Chapter II). Calculation of the volume of distribution (inner and outer pools) was made from plotting the proportion of the injected radioactive cortisol, recovered in these blood samples, on a similar graph (see also Introduction, Chapter II).

Table (15)

Cortisol production rate: control estimations

from single and double compartment models and urine studies

Case	Double compartment			Single Compartment CPR (mg./24 hrs.)	Urine method CPR (mg./24 hrs.)
	volume of distribution (1)		CPR (mg./24 hrs)		
	inner pool	outer pool			
7. M.R.	6.6	16.6	25.8	26.9	22.4
24. M.K.	6.5	20.2	29.5	30.2	27.4

Table 15 shows the results of estimation of cortisol production rate from single and double compartment models, together with that calculated from the urine method, in one subject from the normal group and in another from the 'Cushingoid' group. Estimations from the double compartment model were only 1.1 mg./24 hrs. (4%) and 0.7 mg./24 hrs. (2.3%) lower than estimations from the single compartment model in the first and second cases respectively. Estimations from the double compartment model were still well above those of the urine method. This means, at least in these two cases, that estimations from a single compartment are quite accurate for estimations of control cortisol production rates, and that the presence of more than one compartment does not explain the whole of the discrepancy between the blood and urine method, at least in the control estimations.

Hoet et al (1961) stated that, if more than one compartment exists, the changes of the miscible pool are too slow, in relation to the duration of the experiment (2 hours), to influence the results to any significant extent. No attempt was made in this study to investigate the significance of this factor after ACTH stimulation. Hoet et al (1962) also suggested that in secretion of elevated amounts of cortisol, such as after ACTH stimulation, transcortin is saturated and the free cortisol constitutes an important fraction of the total pool, which is associated with a great increase of volume of distribution. These workers suggested that free cortisol, in these conditions, enters the intracellular space (a second compartment). They concluded that equilibration between the two compartments, if a second one exists, must occur; if this is slow, other calculations must be used, but, if it is rapid, calculation from a single compartment will be quite accurate.

The/

The volume of the inner pool was almost identical in the two cases studied but the volume of the outer pool was larger in the second case, which suggests that a larger volume of distribution of cortisol exists in obese subjects. Peterson (1959 a) reported an inner pool of 6 and an outer pool of 16 litres in normal subjects, and Tait et al (1961) 6 and 13 litres respectively. Hoet et al (1962) reported an outer pool of 14.77 in normal subjects but a 13.45 litre in obese ones, and Gray et al (1962) reported a volume of distribution 67 litres in a case of Cushing's syndrome due to hyperplasia.

8. Repeated assessment of adrenocortical function for the diagnosis of Cushing's syndrome

Case 18 (A.D.) was classified in the 'Cushingoid' group on account of the uncertainty of the clinical features, in spite of a higher than normal control cortisol production rate and a hyper-response to ACTH stimulation. It was decided to observe the patient for a while to see if there was any deterioration, or improvement, in her clinical condition. Five months later, an exacerbation of the clinical condition necessitated re-admission, and tests of cortisol production rate and steroid excretion were repeated to see if there had been any change that would parallel the worsening of the clinical condition.

Examination of table 16 shows that in the second control estimation, the miscible pool and rate of turnover were almost the same as in the first control estimation and the production rate of cortisol by the blood method, which was first slightly higher than normal, dropped to the upper limit of normal. Production rate by the urine method, which was slightly higher than normal on the first occasion, was more abnormal on the second occasion. The urinary 17-KS excretion was almost the same but a marked drop of the total 17-KGS excretion had occurred.

After ACTH, a marked increase of the cortisol production rate by the blood method occurred in the second estimation and the new level was high up in the range observed for other patients with Cushing's syndrome. The miscible pool, which in the first estimation had been high up in the Cushing's range, rose slightly more but the rate of turnover increased markedly in the second estimation. Cortisol production rate by the urine method followed these changes, though to a lesser extent, by increasing from a level well in the/

Table (16)

Cortisol production rate and urinary steroid excretion

repeated estimations after 5 months in case 18 (A.D.)

Test			First estimation*			Second estimation*		
			Control	After ACTH	After dexam.	Control	After ACTH	After dexam.
a.	CPR: blood method							
	Miscible pool	(mg.)	2.1	24.5		2.1	25.8	
	$t_{\frac{1}{2}}$	(min.)	56	68		66	42	
	Rate of turnover	(pool/hr)	0.74	0.61		0.63	0.99	
	Production rate	(mg./24hr)	37.0	358		31.0	614	
b.	CPR: urine method							
	Dose excretion first 24 hr.	(%)	80.8	84.8	91.4	86.9	79.2	81.6
	S.A. THE	(cpm/μg.)	23.3	5.3	53.6	62.4	8.4	893.8
	Production rate	(mg./24hr)	33.2	154	3.41	36.1	268	2.5
c.	17-KS	(mg./24hr)	2.3	5.0	1.0	3.0	14.0	1.0
d.	Total 17-KGS	(mg./24hr)	30.6	118	2.4	16.0	100	2.0

* Dose administered was 1 μc. and 2 μc. in the first and second estimations respectively. ACTH administered in the first estimation was twice daily for two days and once on the morning of the third day, and for the second estimation twice daily for three days; estimations in both cases were carried out on the third day. Dexamethasone was given four times daily, in the first estimation for two days and once on the morning of the third day and in the second estimation for two days; estimations were carried out on the third and second day for the first and second estimations respectively.

the normal range to just above it. Although 17-KS excretion increased nearly three-fold, the new level was still well within the normal control range, and the total 17-KGS excretion even dropped in the second estimation but the level after ACTH stimulation in both cases was much higher than the corresponding control level.

Marked suppression after dexamethasone occurred in all tests, being more marked on the second occasion despite the fact that dexamethasone was given for 8 doses instead of 9 and that estimations were carried out on the second day instead of the third.

The striking difference, therefore, between the first and second estimations was the marked hyper-response in the cortisol production rate to ACTH in the second estimation especially as estimated by the blood method. Because of the worsening of the clinical condition of this patient, supported by the new laboratory findings, a final diagnosis of Cushing's syndrome was agreed upon and it was decided to treat this patient by adrenalectomy. Unfortunately further deterioration of the psychological symptoms occurred, which necessitated transfering the patient to a mental hospital, and surgery was postponed for the time being.

Similar observations were reported by Schteingart et al (1963), who found that cortisol production rate in a patient suspected of having Cushing's syndrome was below the range for this clinical condition. When the test was repeated three months later, the results fell clearly within the range for Cushing's syndrome.

9. Pattern of urinary 17-KS excretion

Table 17 and figure 22 provide information about the daily excretion of 17-KS for the four groups, both under control conditions (repeated estimations) and during ACTH and dexamethasone administration.

(a) Control estimations

The mean in the group of patients with Cushing's syndrome is higher than that of either the normal or obese group and the mean in the obese group is similar or even slightly lower than that of the normal group. Marked overlapping of the ranges is noticed. In individual cases, however, wide variation and marked day-to-day fluctuation occurred mostly in the group with Cushing's syndrome, and to a lesser extent in the 'Cushingoid' group. Less variation and fluctuation occurred in the normal and in the obese groups.

(b) During ACTH administration

Maximum stimulation occurred, in the normal group, on the second and third day. In the group of Cushing's syndrome maximum stimulation occurred on the third day and in one case on the first day. In the 'Cushingoid' group, maximum stimulation occurred mostly on the third day but sometimes also on the second or first day of ACTH administration. In the obese group maximum excretion was noticed on the second day. Again, examination of individual cases showed marked variation and day-to-day fluctuation in the group of Cushing's syndrome and the 'Cushingoid' group, and relatively more uniform patterns in the normal and obese groups. The highest excretory values were recorded for patients with Cushing's syndrome and for the 'Cushingoid' group.

The occurrence of maximum stimulation on the second day in many cases

is/

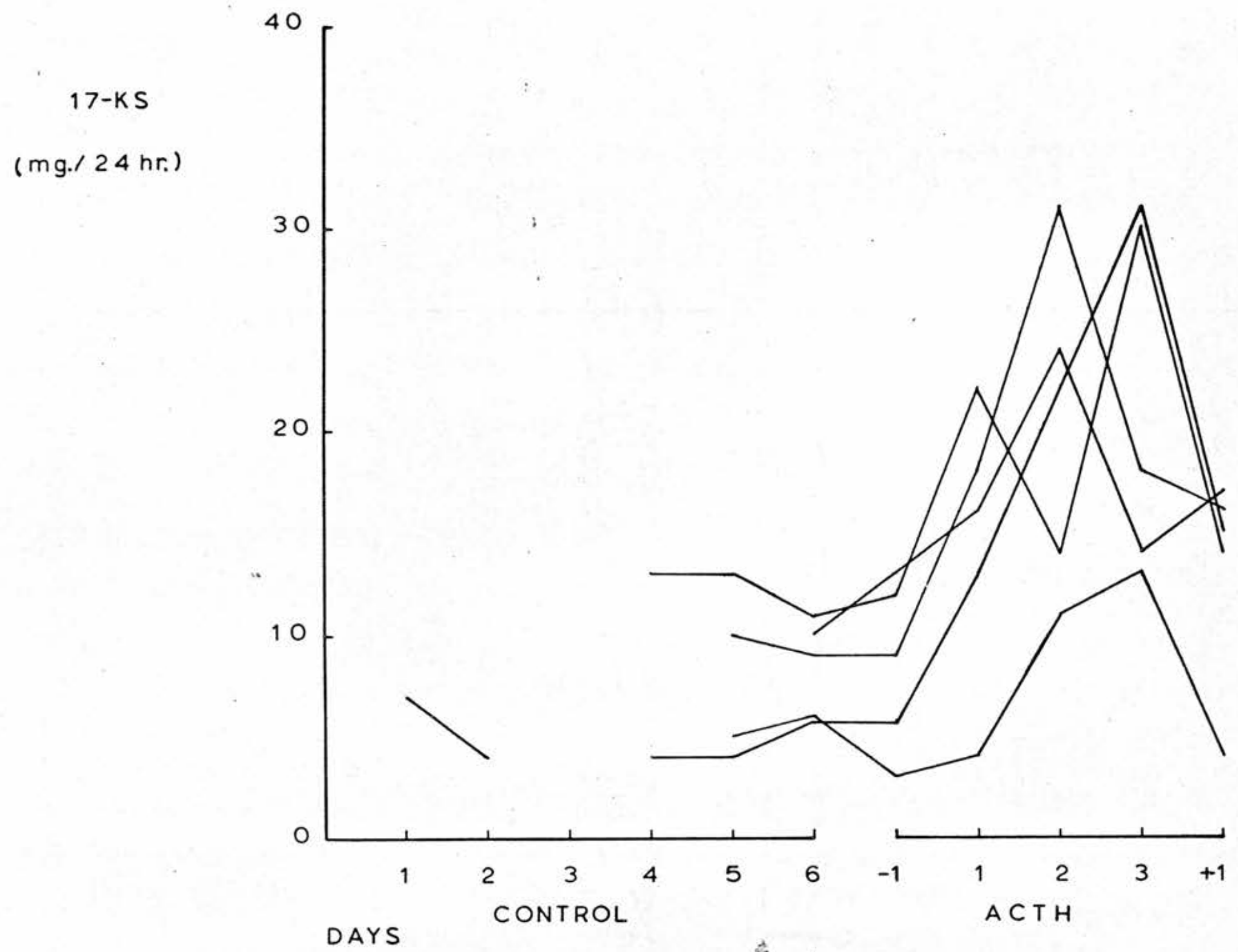

FIGURE 22 a: PATTERN OF URINARY 17-KETOSTEROID EXCRETION IN NORMAL SUBJECTS

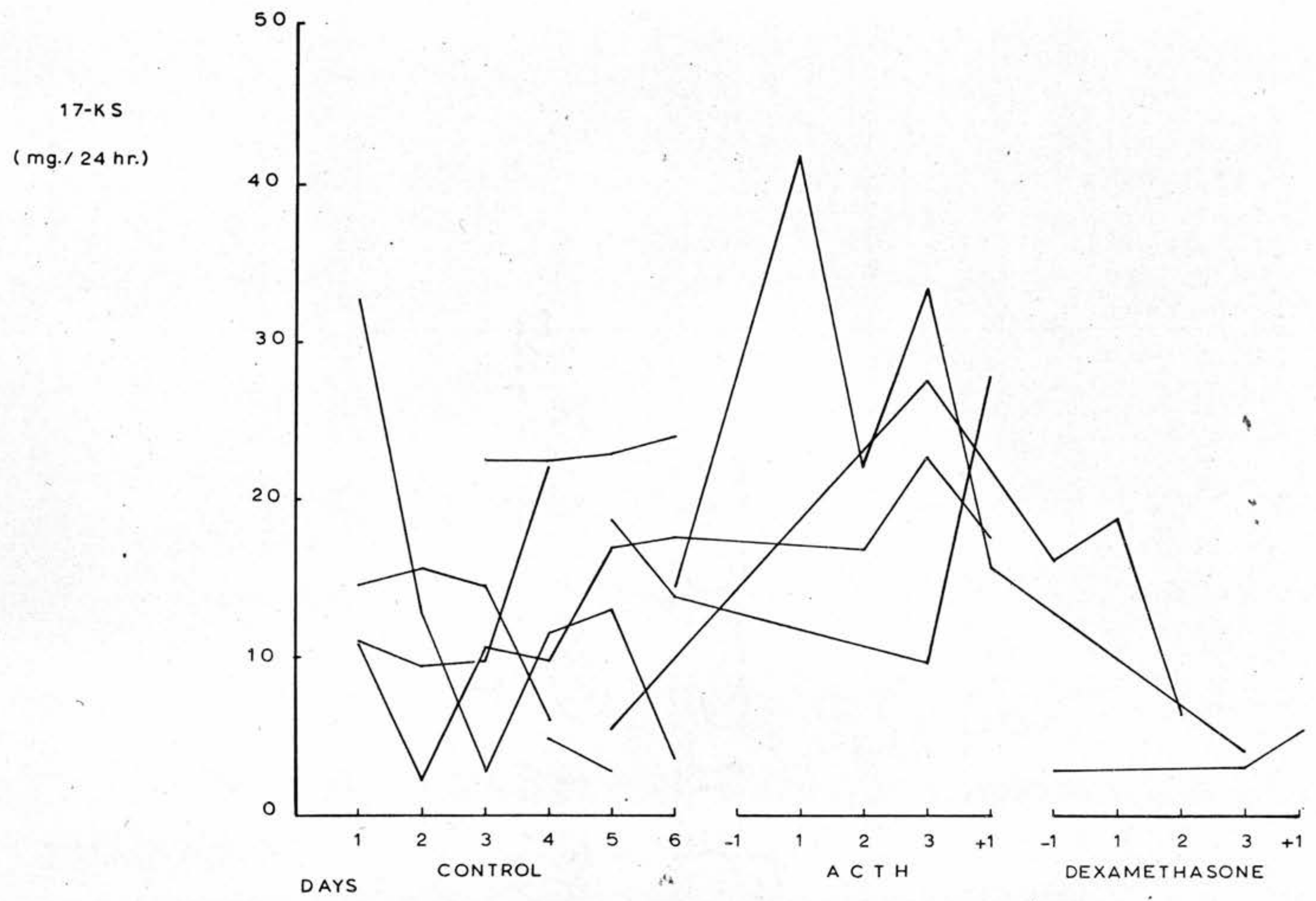

FIGURE 22 b: PATTERN OF URINARY 17-KETOSTEROID EXCRETION IN PATIENTS WITH CUSHING'S SYNDROME

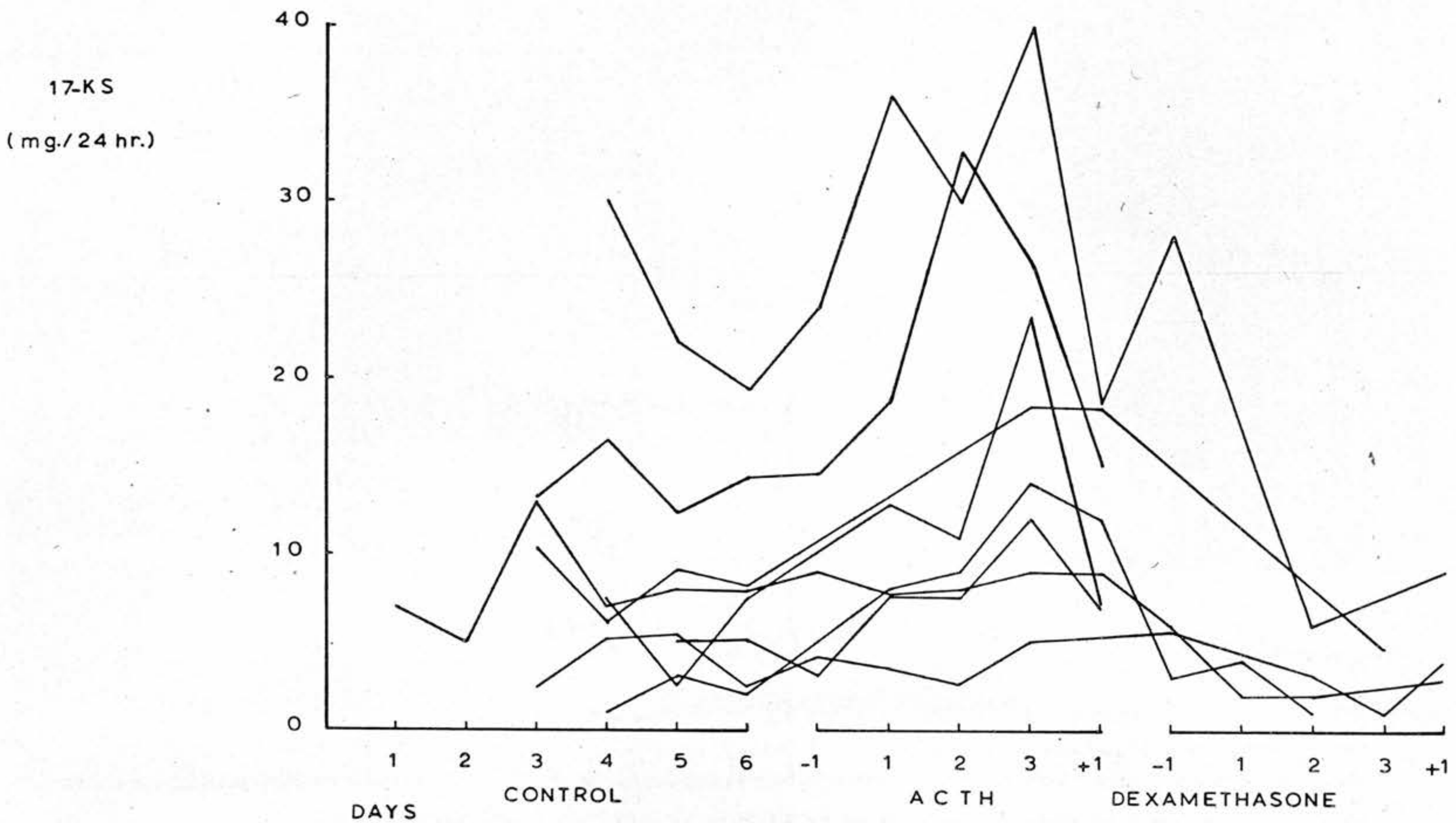

FIGURE 22 c: PATTERN OF URINARY 17-KETOSTEROID EXCRETION IN CUSHINGOID PATIENTS

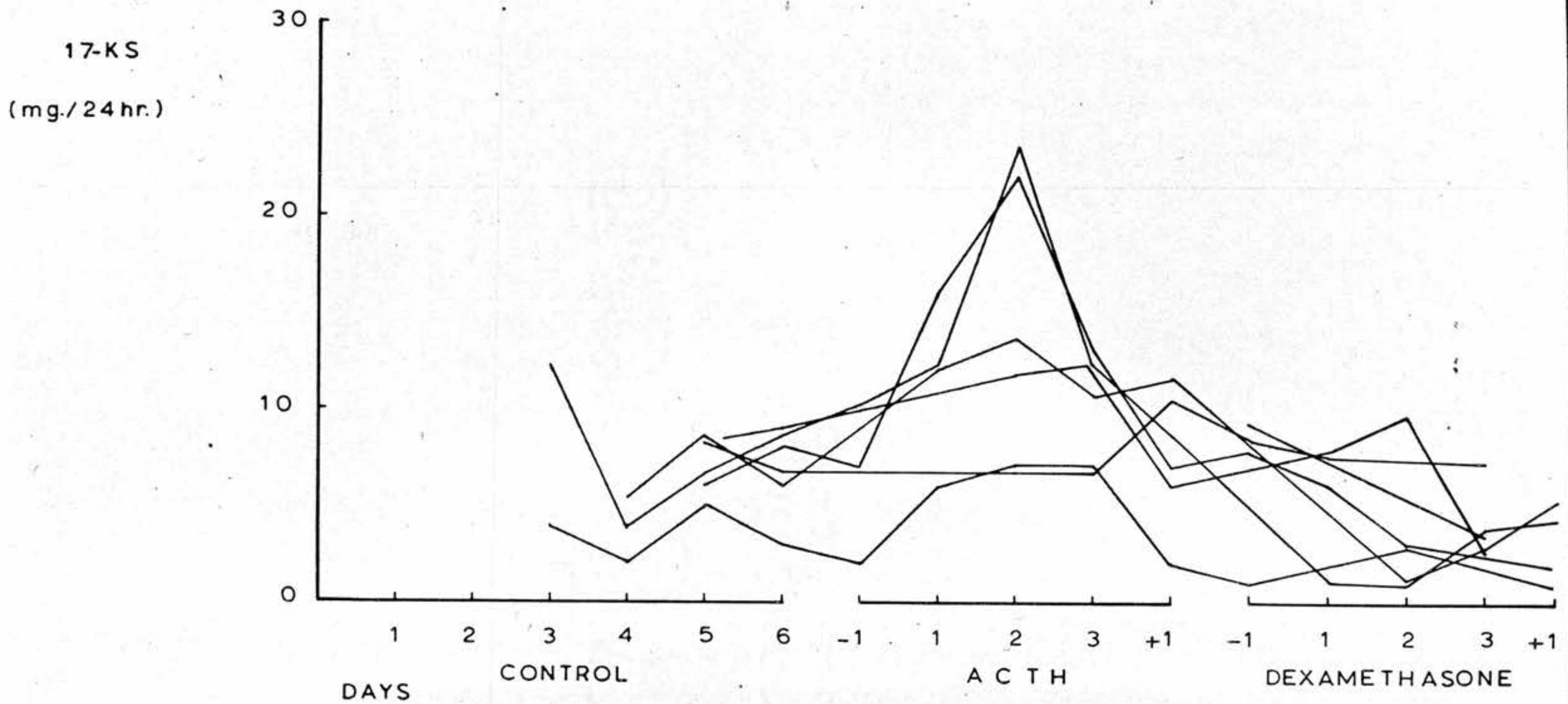

FIGURE 22 d: PATTERN OF URINARY 17-KETOSTEROID EXCRETION IN OBESE SUBJECTS

Table (17)

Pattern of 17-KS excretion under control, stimulation and suppression states

Case	Age (yrs)	Sex	Control estimations						ACTH stimulation (days)					Dexam. suppression (days)				
									-1	1	2	3	+1	-1	1	2	3	+1
1. Normal group																		
2. R.B.	65	M	7.0	4.0														
3. W.J.*	35	M	5.0	6.0					3.0	4.0	11.0	13.0	4.0					
4. W.H.*	55	M	10.0							16.0	24.0	14.0	17.0					
5. M.L.*	42	F	13.0	13.0	11.0				12.0	22.0	14.0	30.0	14.0					
6. M.H.*	35	F	4.0	4.0	6.0				6.0	13.0		31.0	15.0					
7. M.R.*	23	F	10.0	9.0					9.0	18.0	31.0	18.0	16.0					
range	23-65		4.0 - 13.0						6.0-12.0	4.0-22.0	11.0-31.0	13.0-31.0	4.0-17.0					
mean	42.5		7.85						7.5	14.6	20.0	21.2	13.2					
2. Cushing's group																		
a. Operated upon																		
10. J.A.	55	M	18.7	13.8								9.7	27.9					
repeated**			4.8	2.9										2.9			3.0	5.5
13. J.M.	31	M	22.5	22.4	22.9	24.0												
14. G.F.	27	M	32.7	12.9	2.8	11.5	13.1	3.6										
11. J.T.	17	F	14.2							42.0	22.1	33.4	15.7				4.0	
12. M.C.	36	F	5.6	10.2								27.5		16.1	18.6	6.4		
15. H.J.	21	F	14.5	15.6	14.5	6.0												
16. E.S.	22	F	11.0	9.5	9.8	22.3												
b. Not operated upon																		
17. C.M.	18	F	11.1	2.2	10.6	9.8,	16.9	17.6			16.9	22.7	17.6					
range	17-55		2.2 - 32.7								16.9-22.1	9.7-33.4	15.7-27.9	2.9-16.1			3.4-4.0	
mean	28.3		13.23								19.5	23.3	20.4	9.5			3.5	
3. 'Cushingoid' group																		
18. A.D.	19	F	2.4	5.1	5.4	2.3			4.2	3.6	2.7	5.0		5.5		3.2	1.0	3.9
repeated*			1.0	3.0	2.0					8.0	9.0	14.0	12.0	3.0	4.0	1.0	—	1.0
19. W.C.	27	M	10.3	6.1	9.1	8.2						18.4	18.4				4.7	
20. D.M.*	21	M	30.0	22.0	19.0				24.0	36.0	30.0	40.0	18.0	28.0		6.0	—	9.0
21. T.M.	18	M	7.3	2.3	7.4					12.8	10.9	23.3	7.2					
23. D.S.*	34	F	7.0	5.0	13.0	7.0	8.0	8.0	9.0	8.0	8.0	9.0	9.0	6.0	2.0	2.0	—	3.0
24. M.K.*	15	F	13.1	16.3	12.4	14.4			14.6	18.8	32.9	26.4	15.1					
25. D.Y.*	32	F	5.0	5.0					3.0	8.0	8.0	12.0	7.0					
range	15-34		1.0 - 30.0						3.0-24.0	3.6-36.0	2.7-32.9	5.0-40.0	7.0-18.4	3.0-28.0	2.0-4.0	1.0-6.0	1.0-4.7	1.0-9.0
mean	23.7		8.87						10.9	13.6	14.5	18.5	12.4	10.6	3.0	3.1	2.85	4.2
4. Obese group																		
27. R.A.	12	M	5.4	8.6	6.0					10.2	13.7	10.9	11.6			1.2	2.8	5.2
28. T.A.*	17	M	6.0	8.0					7.0	16.0	22.0	13.0	7.0	8.0	6.0	3.0	—	2.0
29. C.S.	14	F	8.2	6.7								6.6	10.4		7.7		7.2	
30. H.L.	31	F	12.2	3.9	6.6	8.6			10.2	12.2	23.5	12.3	8.6		1.1	1.0	3.8	4.3
31. A.I.*	24	F	4.0	2.0	5.0	3.0			2.0	6.0	7.0	7.0	2.0	1.0		3.0	—	1.0
32. C.R.	20	F	9.2														3.7	
33. M.S.	14	F	8.2	8.6							11.7	12.3	6.0		7.4	9.6	2.5	
range	12-31		2.0 - 12.2						2.0-10.2	6.0-16.0	7.0-23.5	6.6-13.0	2.0-11.6	1.0-8.0	1.1-7.7	1.0-9.6	2.5-7.2	1.0-5.2
mean	18.9		6.6						6.4	11.1	15.6	10.4	7.6	4.5	5.6	3.6	4.0	3.1

* Patients received 6 doses of ACTH and 8 doses of dexamethasone (see text), tests were performed on the third and second day respectively. All other patients received 5 doses of ACTH and 9 doses of dexamethasone; tests were performed on the third day on each occasion. ** After unilateral adrenalectomy.

is evidence that estimations carried out on the third day of ACTH administration, using this particular dosage, represent a measure of the maximum capacity of the adrenal cortex.

(c) <u>During dexamethasone administration</u>

Maximum suppression in the group of Cushing's syndrome occurred on the third day, in the 'Cushingoid' group on the second and third days and in the obese group also on the second and third days. The lowest levels were reached by cases in the 'Cushingoid' and obese groups, but a certain degree of variation in each group persisted, indicating that marked suppression did not necessarily occur in every case with this estimation.

10. Pattern of urinary total 17-KGS excretion

Table 18 and figure 23 provide results of estimations of the daily excretion of total 17-KGS for the four groups under control conditions (repeated estimations) and during ACTH and dexamethasone administration.

(a) Control estimations

The ranges and means of the normal and obese groups were almost identical whereas those of the patients with Cushing's syndrome were much elevated. Marked overlapping occurred. Wide variations and day-to-day fluctuations occurred in the group of Cushing's syndrome and to a lesser extent in the 'Cushingoid' group. Only slight variation and fluctuation occurred in the normal and obese groups.

Day-to-day fluctuations in the level of 17-KGS excretion of patients suffering from Cushing's syndrome were reported by Birke et al (1956), Bassöe et al (1958), Dyrenfurth et al (1960), Ekman et al (1961), Prunty (1961), Slater et al (1962) and Brooks et al (1963). The last-named authors observed levels of 11.0 and 40.0 mg./day in one patient for 17-KGS and 14.0 and 20.0 for 17-KS, and Prunty (1961) reported similar fluctuations in plasma cortisol and cortisol production rate. These observations could explain the occasional finding of normal results in Cushing's syndrome, especially among less sensitive tests such as the urinary steroid excretion tests.

Rosner et al (1963) observed normal daily variations of steroid excretion in obese subjects, and this observation is supported by the findings of the present study.

(b) During ACTH stimulation

Maximum excretion occurred on the third day in the normal group

and/

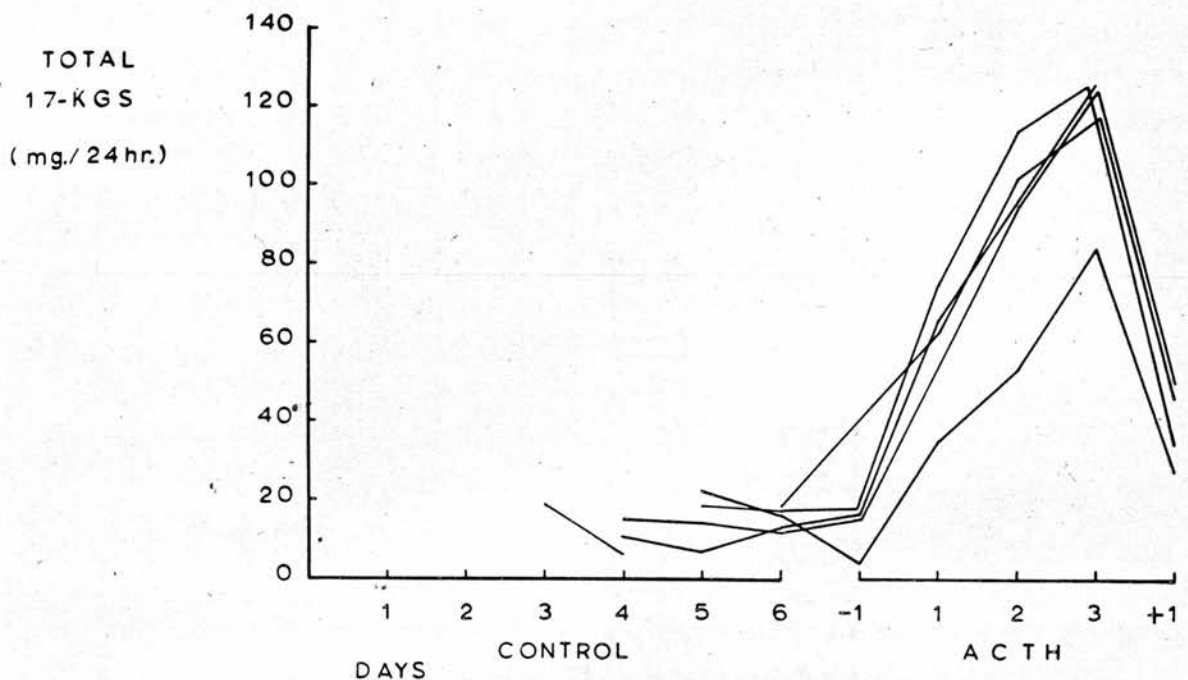

FIGURE 23 a: PATTERN OF URINARY TOTAL 17-KETOGENIC STEROID EXCRETION IN NORMAL SUBJECTS

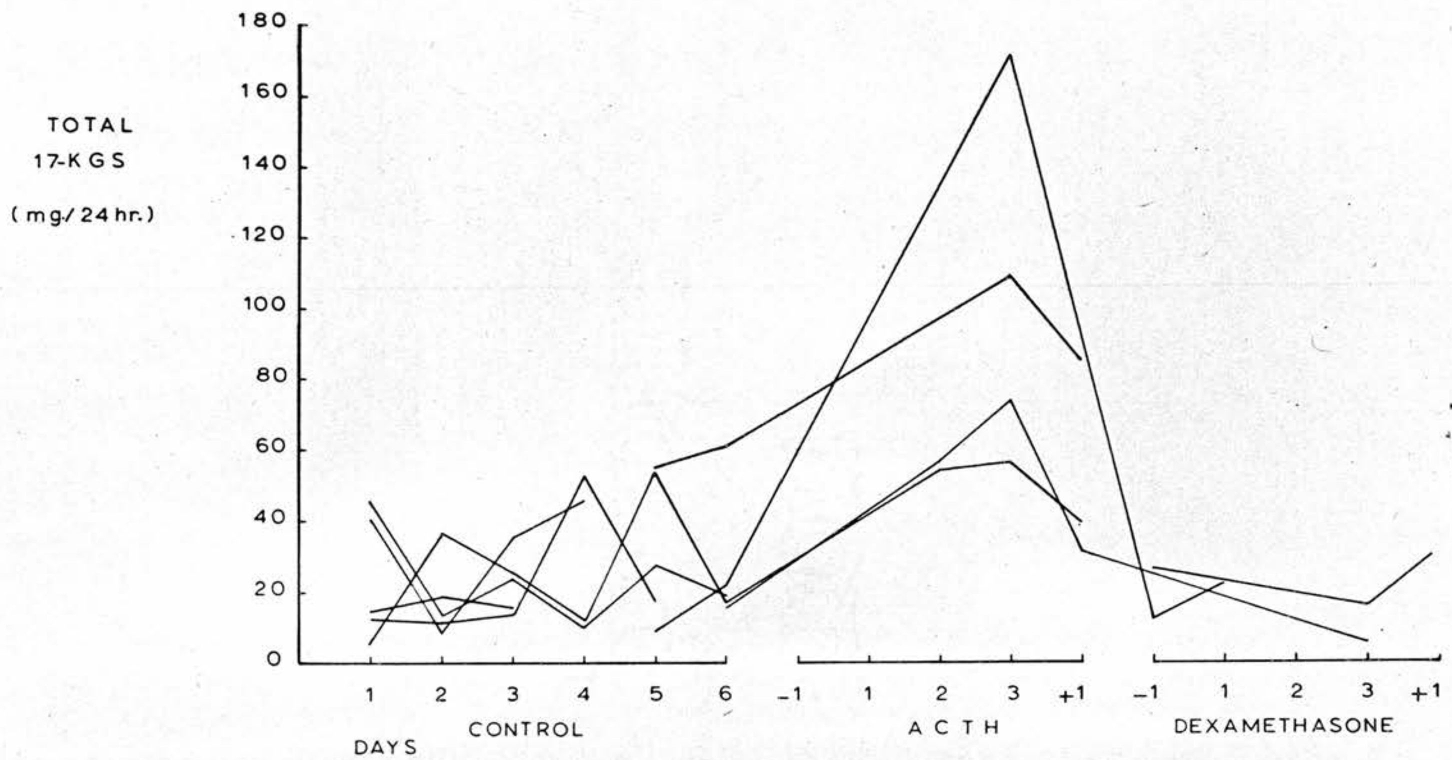

FIGURE 23 b: PATTERN OF URINARY TOTAL 17-KETOGENIC STEROID EXCRETION IN PATIENTS WITH CUSHING'S SYNDROME

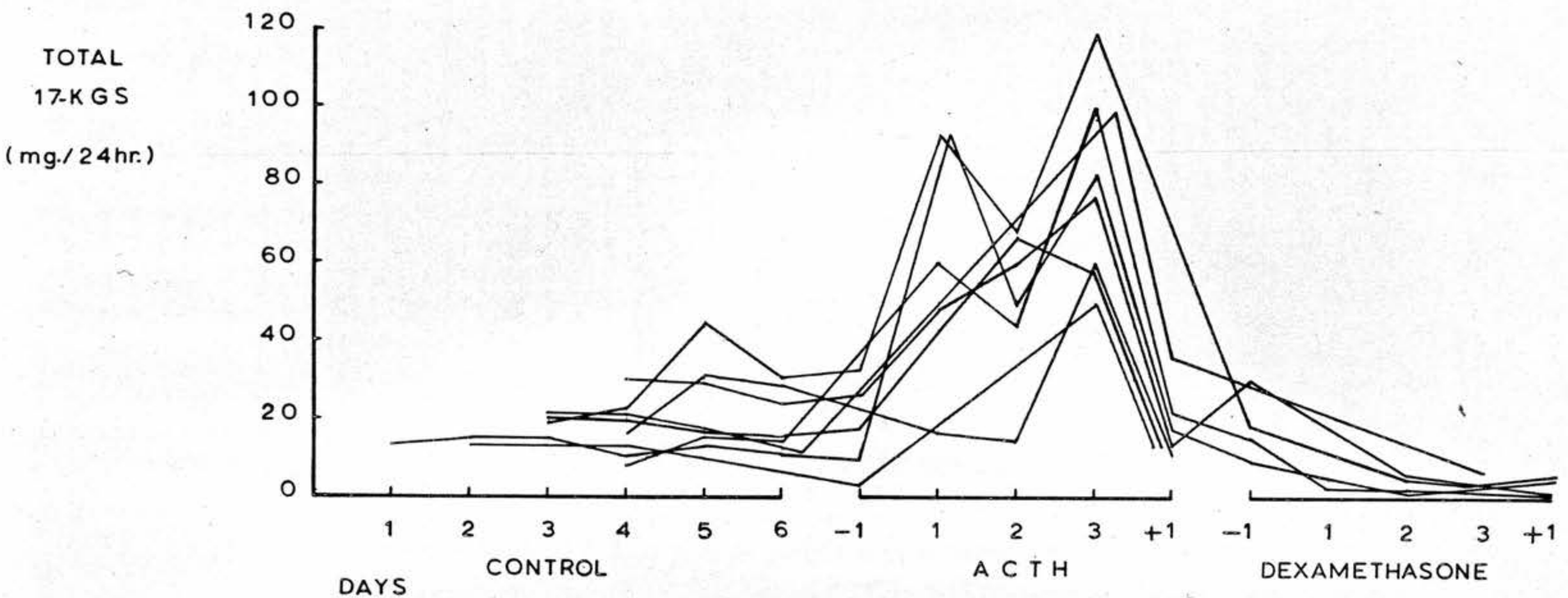

FIGURE 23c: PATTERN OF URINARY TOTAL 17-KETOGENIC STEROID EXCRETION IN CUSHINGOID PATIENTS

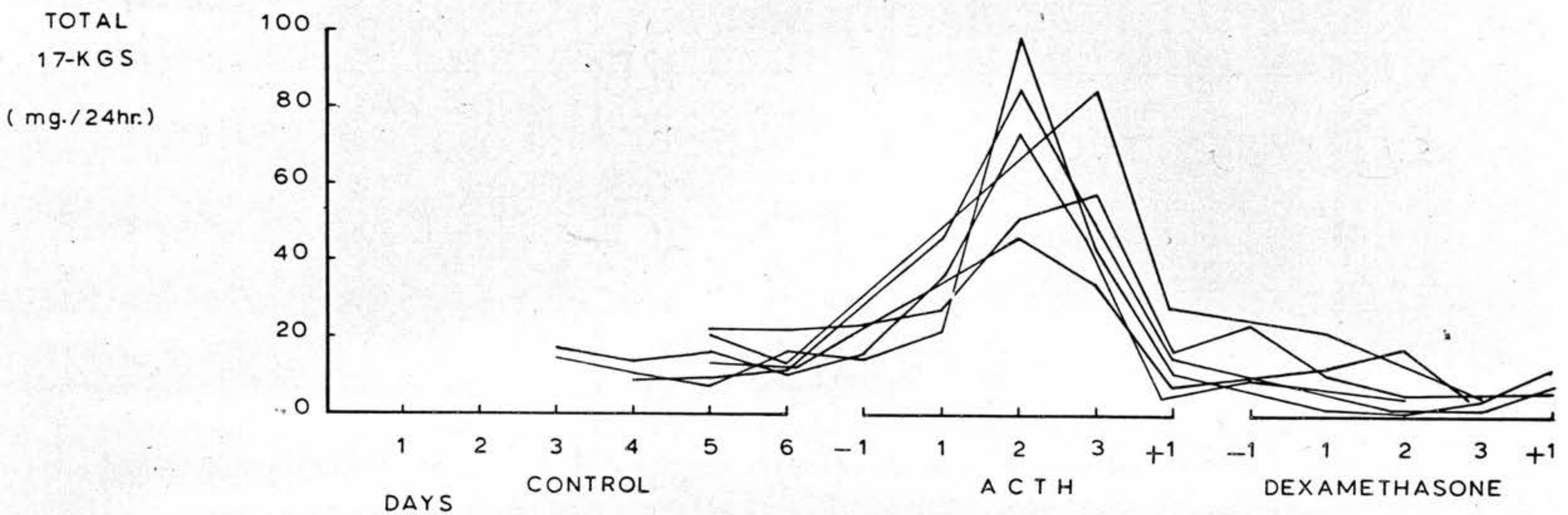

FIGURE 23 d : PATTERN OF URINARY TOTAL 17-KETOGENIC STEROID EXCRETION IN OBESE SUBJECTS

Table (18)

Pattern of total 17-KGS excretion under control, stimulation and suppression states.

Case	Age (yrs)	Sex	Control estimations						ACTH stimulation (days) -1	1	2	3	+1	Dexam. suppression (days) -1	1	2	3	+1
1. Normal group																		
2. R.B.	65	M	19.0	6.0														
3. W.J.*	35	M	22.0	16.0					4.0	35.0	53.0	84.0	27.0					
4. W.H.*	55	M	18.0							63.0	102.0	117.0	46.0					
5. M.L.*	42	F	15.0	14.0	12.0				15.0	(gl.)	95.0	124.0	50.0					
6. M.H.*	35	F	10.0	7.0	13.0				15.0	64.0		125.0	(gl.)					
7. M.R.*	23	F	18.0	17.0					16.0	75.0	114.0	125.0	34.0					
range	23-65		6.0 - 22.0						4.0-16.0	35.0-75.0	53.0-114.0	84.0-125.0	27.0-50.0					
mean	42.5		14.4						12.5	59.3	91.0	115.0	39.3					
2. Cushing's group																		
a. operated upon																		
10. J.A. **	55	M	54.9	60.6								109.0	85.1					
(repeated)			20.3	26.4										26.4			15.7	29.6
13. J.M.	31	M	40.5	8.3	35.3	45.8												
14. G.F.	27	M	45.4	13.6	24.0	10.8	27.3	18.4										
			(gl.)	(gl.)	(gl.)	(gl.)	(gl.)	(gl.)										
11. J.T.	17	F	15.8							64.0	56.7	73.0	31.1				5.2	
12. M.C.	36	F	8.7	21.8								176.0		12.5	22.1			
15. H.T.	21	F	14.2	18.2	15.8													
16. E.S.	22	F	12.7	11.6	13.4	52.1	17.7											
b. not operated upon																		
17. C.M.	18	F	5.7	36.2	25.7	12.2	54.4	15.5			54.4	56.0	39.8					
range	17-55		8.3 - 60.6								54.4-56.7	56.0-176.0	31.1-85.1	12.5-26.4			5.2-15.7	
mean	28.3		25.3								55.5	103.0	19.5	19.5			10.5	
3. 'Cushingoid' group																		
18. A.D.	19	F	18.1	22.6	44.9	30.6			32.8	93.1	67.8	118.0		18.2		4.5	2.4	6.8
repeat*			8.0	16.0	14.0					60.0	44.0	100.0	22.0	15.0	2.0	2.0	—	1.0
19. W.C.	27	M	20.7	21.1	18.0	12.3						99.0	35.6				6.7	
20. D.M.*	21	M	30.0	29.0	24.0				26.0	48.0	60.0	77.0	13.0	30.0		5.0		2.5
21. T.M.	18	M	16.7	30.5	28.9					16.5	14.6	59.0	10.8					
23. D.S.*	34	F	13.0	15.0	15.0	10.0	13.0	11.0	10.0	93.0	49.0	83.0	17.0	9.0	5.0	1.0	—	5.0
24. M.K.*	15	F	20.4	19.7	16.9	15.8			17.2	42.5	67.8	58.0	13.4					
25. D.Y.*	32	F	13.0	13.0					3.0	(gl.)	(gl.)	50.0	13.0					
range	15-34		8.0 - 44.9						3.0-32.8	16.5-93.1	14.6-67.8	50.0-118.0	10.8-35.6	9.0-30.0	2.0-5.0	1.0-5.0	2.4-6.7	1.0-6.8
mean	23.7		19.4						17.8	58.9	50.5	80.7	17.8	18.1	3.5	3.1	3.5	3.8
4. Obese group																		
27. R.A.	12	M	8.6	9.5	11.8					45.5	84.6	48.0	14.4			1.0	1.1	7.4
28. T.A.*	17	M	22.0	22.0					23.0	27.0	51.0	57.0	16.0	23.0	10.0	5.0	—	6.0
29. C.S.	14	F	20.4	13.0								84.0	26.6		20.4		4.3	
30. H.L.	31	F	14.5	9.7	7.3	16.3			14.3	21.5	98.3	42.0	10.8		1.1	1.0	4.2	11.4
31. A.I.*	24	F	17.0	14.0	16.0	11.0			15.0	35.0	73.0	48.0	4.0	9.0		5.0	—	6.0
32. C.R.	20	F	11.7														2.5	
33. M.S.	14	F	12.9	11.0							45.5	33.0	6.9		11.9	17.0	3.8	
range	12-31		7.3 - 22.0						14.3-23.0	21.5-45.5	45.5-98.3	33.0-84.0	4.0-26.6	9.0-23.0	1.1-20.4	1.0-17.0	1.1-4.3	6.0-11.4
mean	18.9		13.8						17.4	32.3	70.5	52.0	13.1	16.0	10.9	5.8	3.2	7.7

* Patients received 6 doses of ACTH and 8 doses of dexamethasone (see text) tests were performed on the third and second day respectively. All other patients received 5 doses of ACTH and 9 doses of dexamethasone; tests were performed on the third day on each occasion.

** After unilateral adrenalectomy.

gl. refers to glycosuria.

and in the group of Cushing's syndrome. In the 'Cushingoid' group maximum excretion mostly occurred on the third day, but sometimes on the second day, and on three occasions a first peak was noticed on the first day followed by a drop on the second day and a higher peak on the third day. In the obese group, maximum excretion occurred mostly on the second day but sometimes on the third day. Although the highest excretion value was reached by a patient with Cushing's syndrome, the mean of the normal group on the third day was higher than the corresponding mean of any of the other groups. Overlapping of the ranges is noticed, but wide variations and fluctuations are not observed, except to some extent in the 'Cushingoid' group.

The occurrence of maximum excretion on the second day in many cases is further evidence that estimations carried out on the third day of ACTH stimulation, using this particular dosage, represent a measure of the maximum capacity of the adrenal cortex.

An interesting observation in the study of the total 17-KGS pattern in response to ACTH stimulation, was the detection of glycosuria in two subjects of the normal group (Case 5, M.L. and case 6, M.H.) and one subject of the 'Cushingoid' group (case 25, D.Y.) during the first, second, or third day of ACTH administration or even on the following day. Glycosuria was detected also in the urine of one patient with Cushing's syndrome (patient 14, G.F.) both when showing high levels of total 17-KGS excretion, and when levels were well within the normal range. The interpretation of these observations could be that excess ACTH, rather than cortisol, is sometimes directly responsible for the occurrence of glycosuria and that glycosuria, and indeed/

indeed Cushing's syndrome, are sometimes due to faults in the ACTH - secreting mechanism, i.e. of pituitary or hypothalamic origin, rather than of adrenal origin.

Other indirect evidence that the cause of this clinical condition lies in the pituitary rather than in the adrenals was offered by Jailer (1962) who, commenting on the hyper-sensitivity to ACTH characteristic of this syndrome, stated that this hyper-sensitivity existed in patients with pituitary tumours just as it did in those with bilateral adrenal hyperplasia as the only pathology. He stated that hypersenistivity to ACTH disappeared after successful treatment of the pituitary, by irradiation, and furthermore, when the disease recurred a few years later, the characteristic hyper-sensitivity to ACTH once again developed. Jailer concluded that these data are compatible with the hypothesis that Cushing's syndrome with adrenal hyperplasia is primarily a pituitary disease due to secretion perhaps of an abnormal ACTH, or another abnormal hormone, which potentiates the action of ACTH.

Further evidence pointing to the same conclusion was offered by Nugent et al (1960). These workers stated that the pathogenesis of Cushing's syndrome associated with administration of glucocorticoids or ACTH or with tumours of the adrenal cortex was readily apparent. They postulated that the syndrome, when associated with adrenal hyperplasia, was the result of increased secretion of ACTH, which satisfactorily explains the bilateral adrenal hyperplasia, the increased responsiveness of the adrenal glands to ACTH stimulation and the increased amounts of adrenal steroids secreted by the glands, found in the blood or excreted in urine. This postulate, they stated,/

stated, is also compatible with the fact that therapeutic success has been achieved in some cases following hypophysectomy or irradiation of the pituitary gland, but would not be compatible with reports that no increase would be demonstrated in blood levels of ACTH in cases of Cushing's syndrome associated with adrenal hyperplasia. These workers then administered ACTH to normal subjects in amounts too small to lead to detectable concentrations of blood ACTH, (viz. 1.5 - 5.0 I.U. ACTH intravenously per day for 4 days). They proved that, under such circumstances, constant elevation of plasma 17-hydroxycorticosteroids, with loss of diurnal variation and increased responsiveness to maximum stimulation by ACTH, occurred in these normal subjects. These workers, therefore, concluded that, (1) the possibility that Cushing's syndrome associated with adrenal hyperplasia might result from increased secretion of ACTH could not be excluded on the grounds that increased blood levels of ACTH were not demonstrable, (2) patients with Cushing's syndrome and adrenal hyperplasia might secrete ACTH at an abnormally high rate during at least part of the day, (3) the site of this pituitary "dribbling" of ACTH is not known; it may be in the hypothalamus, the pituitary, in the interconnecting pathways or in some other controlling mechanisms.

The hypothesis that sudden increases of adrenal activity occur in the form of "bouts", whether due to adrenal or pituitary cause, rather than the hypothesis of "dribbling" of the pituitary is supported by other workers. Thus Prunty (1961) showed that, in Cushing's syndrome due to hyperplasia, in addition to changing levels of 17-KGS, spontaneous fluctuations of plasma cortisol and CPR, gross discrepancies occurred between the plasma cortisol level/

level and the response to ACTH; for instance, a low value for the plasma cortisol level was associated with a very high response to ACTH stimulation. Prunty stated that such lability prompted the question whether some patients developed their symptoms as a result of sudden increases of adrenal secretion during their daily lives, whilst measurements made under basal conditions in hospital gave a falsely low idea of their adrenal activity.

The mechanism by which glycosuria occurs in the presence of excess ACTH is not known. It might be due to a direct effect on the β-cells of the islets of Langerhans of the pancreas.

(c) During dexamethasone administration

Maximum suppression occurred in the group of Cushing's syndrome on the third day, in the 'Cushingoid' group on the second and third days and in the obese group mostly on the third day but sometimes on the second day. Marked suppression on the third day was observed in all cases except patient J.A. in the group with Cushing's syndrome. This, together with the fact that suppression on the second day was almost equally marked in many cases and that plasma cortisol level was too low to be estimated on the third day, justified carrying out all the tests on the second day of dexamethasone administration instead of the third day. Excretion of 5.0 mg./24 hrs., or less of total 17-KGS, was again noticed on the second day in some cases of the 'Cushingoid' and obese groups. More estimations in these two groups and in the normal subjects and patients with Cushing's syndrome await further work, to see if there will be any significant separation of the levels reached in the four groups.

B. Simultaneous estimation of cortisol production rate, plasma cortisol and plasma-protein binding of cortisol

1. Control estimations

Table 19* provide the results obtained by the in vitro method (see also figure 24 - 26, table 28 and 29). The in vivo method was also carried out in one normal and two 'Cushingoid' subjects.

(a) Total plasma cortisol

The highest level was recorded by a patient in the 'Cushingoid' group. The level attained by the patient with Cushing's syndrome came next and was higher than any of those in either the normal or obese groups. The lowest mean was that of the obese group and the lowest single level was also recorded by a patient of the obese group. The level reached by the patient with Cushing's syndrome is significantly higher than the mean of the normal group. Because of the small number of cases studied in the group of Cushing's syndrome and in the obese group, the difference between the level of the patient of the former group and the means of the two patients of the latter group is not significant. The level of total plasma cortisol follows more or less the cortisol production rate but the relationship is not linear.

The normal range and mean reported in this study agrees very well with those reported by other workers (see table 4),although some authors presented higher means or wider ranges. For instance, Peterson and Wyngaarden (1956) reported a range of 4.0 - 24.0 µg./100 ml., Waxman et al (1961) 2.1 - 22.6 µg./100 ml., Layne et al (1962) 13.7 ± 4.3 (S.E.) µg./100 ml., Beck et al (1962) 3.0 - 28.0 µg./100 ml. and Nugent et al (1963) 8.0 - 29.0 µg./100 ml. Similarly, most workers/

*Ranges and means of CPR estimations are not given. These are provided in table 9 for a larger number of subjects in each group.

Table (19)
Simultaneous determination of CPR, plasma level and plasma-protein binding of cortisol
Control estimations

Case	Age (yrs)	Sex	CPR (mg./24hr)	Total plasma cortisol (μg./100ml.)	NPB cortisol in vitro (%)	NPB cortisol in vitro (μg./100ml)	NPB cortisol in vivo (%)	NPB cortisol in vivo (μg./100ml)
1. Normal group								
2. R.B.	65	M	30.0	15.9	11.1	1.75		
3. W.J.	35	M	17.5	9.7	14.1	1.37		
4. W.H.	55	M	31.2	13.4	16.0	2.15		
5. M.L.	42	F	21.8	9.3	14.8	1.38		
6. M.H.	35	F	28.8	7.9	13.0	1.00		
7. M.R.	23	F	26.9	15.4	15.5	2.39	8.9	1.37
range	23-65			7.9-15.9	11.1-16.0	1.00-2.39		
mean	42.5			11.9	14.1	1.67		
S.D.				3.41	1.79	0.52		
S.E.				1.39	0.73	0.21		
2. Cushing's group								
10. J.A.*	55	M	52.8	23.3	17.9	4.2		
3. 'Cushingoid' group								
18. A.D.	19	F	37.0	15.2	23.0	3.50		
19. D.M.	21	M	45.6	29.6	17.5	4.70		
23. D.S.	34	F	18.7	10.6	15.4	1.62		
24. M.K.	15	F	30.2	14.3	10.2	1.46	9.7	1.39
25. D.Y.	32	F	26.4	9.3	17.7	1.64	12.4	1.15
range	15-34			9.3-29.6	10.2-23.0	1.46-4.70	9.7-12.4	1.15-1.39
mean	24.2			15.8	16.8	2.58	11.1	1.27
S.D.				8.09	4.62	1.44	1.91	0.16
S.E.				3.62	2.06	0.64	1.35	0.11
4. Obese group								
28. T.A.	17	M	22.1	6.4	24.4	1.55		
31. A.I.	24	F	21.4	16.8	12.5	2.10		
range	17-24			6.4-16.8	12.5-24.4	1.55-2.10		
mean	20.2			11.6	18.5	1.83		
S.D.				7.36	8.41	0.39		
S.E.				5.20	5.90	0.27		

* 1 μc. was given to this patient and estimations carried out after unilateral adrenalectomy. All other patients received 2 μc.

workers have observed normal or lowered means in obese subjects, which agree with the findings of the present study. Thus Poisnick and DiRaimondo (1956) reported that plasma 17-hydroxycorticosteroids were within normal limits in a group of obese women in spite of the fact that excretion of 17-hydroxycorticosteroids was increased in some of them. Szenas and Pattee (1959) observed lowered plasma cortisol levels in obese subjects (5.9 ± 0.58, S.D., μg./100 ml. against 12.14 ± 0.6 μg./100 ml. in normal subjects), and reported a more rapid disappearance of cortisol from blood than in normal subjects, from which they suggested that the low plasma cortisol levels in obesity might be due to either (a) diminished CPR (b) greater dilution by increased volume of body fluids or tissues (i.e. a larger volume of distribution), or (c) increased removal of cortisol (which could be a reflection of either increased metabolic conversion, increased excretion or absorption by body depots).

Hoet et al (1962) reported a mean level of plasma cortisol of 10.1 μg./100 ml. in obese against 10.6 μg./100 ml. in normal subjects. From calculations of the volumes of distribution, they concluded that cortisol has a volume of distribution approaching the volume of the extracellular space, and accordingly that the apparent volume of distribution must be influenced by the same factors as this space. Therefore, these workers suggested that, with an increased volume of distribution, a steady CPR and a steady rate of turnover, plasma cortisol levels must be diminished. Mlynaryk et al (1962) similarly reported decreased plasma levels of cortisol in obese subjects. Gogate and Prunty (1963), however, found a range of plasma cortisol concentration of 5.2 - 22.3 μg./100 ml. in an obese group against 5.0 - 16.0 μg./100 ml. in normals, but only in

2/

2 cases was the upper limit of normal exceeded.

In Cushing's syndrome, Sweat (1955) reported plasma cortisol levels up to 135 μg./100 ml. Doe et al (1960 b) found a mean of 32.7 μg./100 ml. in 4 cases due to bilateral hyperplasia (normal mean 17.2) and, in another report, Doe et al (1960 a) reported a mean of 27.0 μg./100 ml. against a normal range 4.0 - 26.0 μg./100 ml. (mean 13.6). These workers observed a diurnal variation in the normal group, which was lost in the patients with Cushing's syndrome. Dyrenfurth et al (1960) obtained a mean of 20.2 μg./100 ml. in Cushing's syndrome, against 14.3 μg./100 ml. and 13.8 μg./100 ml. for normal males and females respectively. Gray et al (1962) obtained a level of 55 μg./100 ml. in a case of Cushing's syndrome and Doe et al (1963), using radioisotopic cortisol to correct for losses during the experiment, obtained a mean of 27.7 μg./100 ml. in 4 cases of Cushing's syndrome against 18.1 μg./100 ml. in normal subjects.

On the other hand Prunty (1961) doubted the value of this test in diagnosing Cushing's syndrome. He obtained a range of plasma cortisol of 3.0 - 56.0 μg./100 ml. in 12 cases of Cushing's syndrome, but in only 6 of them was the upper limit of normal exceeded. He also reported fluctuations of levels from time to time. Brooks et al (1963) supported this view, as they obtained a range of 3.0 - 25.0 μg./100 ml. in adrenal hyperplasia and 15.0 - 26.0 μg./100 ml. in adrenal adenoma compared with 5.0 - 16.0 μg./100 ml. in normal subjects. Tait (1963) stated that for steroids with a low clearance, such as cortisol, it is to be expected that there will be an appreciable lag in the response of the plasma concentration to variations in secretion rate. This, together with the influence of variable volumes of distribution and fluctuations of CFR, as reflected in plasma levels of

cortisol/

cortisol, might explain some of the unsatisfactory results just mentioned.

The value of this test, however, seems to be considerable in the differential diagnosis of Cushing's syndrome from obesity. Thus, Hoet et al (1961) reported a range of 17.4 - 27.1 µg./100 ml. in Cushing's syndrome due to adrenal hyperplasia as compared with a mean of 9.8 ± 3.4 (S.E.) µg./100 ml. and 9.7 ± 2.4 µg./100 ml. in obese and normal subjects respectively. Similarly, Schteingart et al (1963) found a mean of 28.3 ± 3.85 (S.E.) µg./100 ml. in Cushing's syndrome against 14.36 ± 3.12 µg./100 ml. and 25.2 ± 2.46 µg./100 ml. in obese and normal groups respectively. The mean in the obese group was significantly lower than that of the normal group and actually 8 of the 14 patients gave values below the lowest normal.

(b) Non-protein-bound cortisol

i- Expressed as "percent of the total plasma cortisol". Although, the level reached by the patient with Cushing's syndrome is higher than any of the normal group, marked overlapping occurs among the group of Cushing's syndrome, the 'Cushingoid' group and obese groups, and no significant difference occurred between any two of these groups.

These figures are higher than those reported by Mills et al (1959), who found 5% unbound cortisol at plasma concentrations of 10 µg./100 ml., 20% at 40 µg./100 ml. and 28% at 80 µg./100 ml. Mills et al (1960) reported an average of 10% unbound cortisol in normal subjects. Layne et al (1962), however, reported a much lower degree of binding in normal subjects, namely 78% ± 3.7 (S.E.). Gray et al (1962) comparing normal subjects and patients with/

with Cushing's syndrome reported a level of 37% unbound cortisol in a case of Cushing's syndrome due to adrenal hyperplasia against a range of 4.0 - 10.0% in normal subjects.

ii- Non-protein-bound cortisol, expressed as its absolute value, i.e. in μg./100 ml. The highest levels are reached by the patient with Cushing's syndrome and by one of the patients in the 'Cushingoid' group, and there is a wide separation between the level of the Cushing's patient on one hand and all the subjects in either the normal or obese groups on the other. There is a highly significant difference between the level in the patient with Cushing's syndrome and the mean of the normal group. Again because of the small number of cases in the group of Cushing's syndrome and the obese group, the difference between the level of the patient of the former group and the means of the two subjects of the latter group was not significant.

Doe et al (1960 b) reported a mean of 16.6 μg. NPB/100 ml. plasma in 4 cases of Cushing's syndrome due to bilateral hyperplasia against 0.9 μg./100 ml. in 11 normal subjects, and Gray et al (1962) reported a level of 20.0 μg./100 ml. in one patient with Cushing's syndrome due to adrenal hyperplasia against a normal range of 0.39 - 1.28 μg./100 ml. (mean 0.67). Recently, Doe et al (1963), adding radioisotopic cortisol to plasma and using a dialysis technique at 37.5°C, obtained a mean of 6.0 μg./100 ml. in 4 cases of Cushing's syndrome against a range of 1.1 - 2.7 μg./100 ml. (mean 1.8) in normal subjects. These results agree very well with those reported in the present study. When NPB cortisol was expressed as μg./100 ml., Doe et al observed circadian rhythm in the normal group but not in patients with Cushing's/

Cushing's syndrome; the mean percentage of NPB cortisol remains constant during the 24 hours. This again agrees with the findings in the present study, and confirms the superiority of expressing results as μg. NPB cortisol/ 100 ml. instead of as a percentage of the total plasma cortisol.

In the *in vivo* method lower values were observed than the *in vitro* method. The difference between the two methods was sometimes marked.

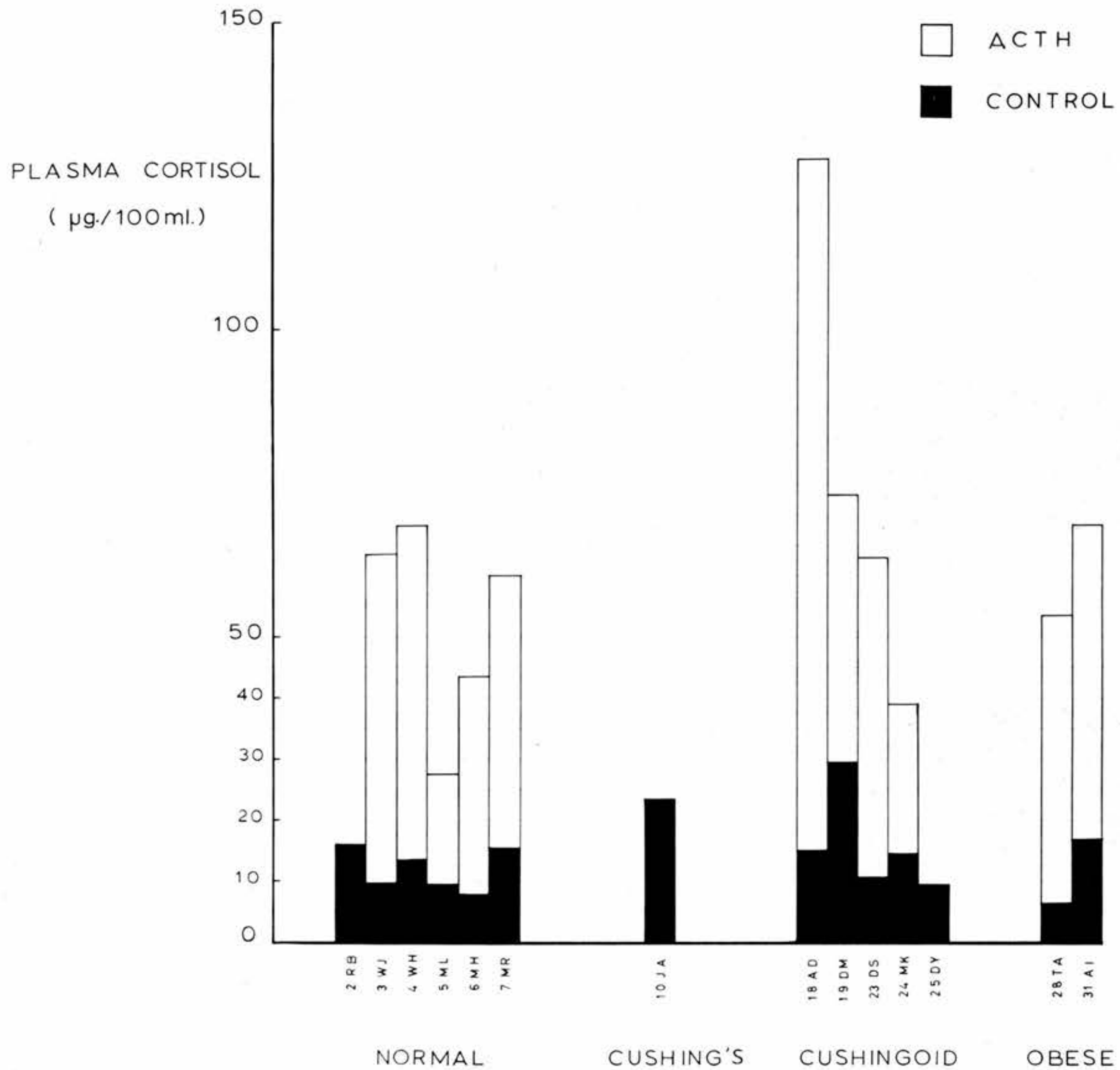

FIGURE 24: PLASMA CORTISOL

CONTROL ESTIMATIONS AND ESTIMATIONS AFTER ACTH

2. Estimations after ACTH stimulation

Table 20* provides the results obtained by the in vitro method on the morning of the third day of ACTH stimulation in subjects from the normal, 'Cushingoid' and obese groups (see also figures 24 - 26). The in vivo method was simultaneously carried out in three subjects of the normal group and one patient of the 'Cushingoid' group.

(a) Total plasma cortisol

The highest level was reached by a patient in the 'Cushingoid' group and the ranges of the normal and obese groups were almost similar. There is no significant difference between any two of these means. The relationship between cortisol production rate and total plasma cortisol by this method was less clear after ACTH. The difference between the mean of total plasma cortisol concentration of each group and its corresponding control mean was, however, highly significant.

Expressing the results of total plasma cortisol as "increase over the control level" in µg., the highest level was observed in the 'Cushingoid' patients, but an overlap occurred between the levels in the three groups and there was no significant difference between the means. Similarly, when results were expressed as "percent increase over the control level", there was marked overlapping between the ranges found in the three groups and no significant difference between them.

No detailed comparison could be made with figures of other workers, because of the different dosage of ACTH used in this study. Most workers, however, obtained significant/

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* Ranges and means of CPR estimations are not given. These are provided in table 11 for a larger number of subjects in each group.

Table (20)
Simultaneous determination of CPR, plasma level and plasma-protein binding of cortisol
Estimations after ACTH

Case	Age	Sex	CPR	Total plasma cortisol	NPB Cortisol			
					in vitro		in vivo	
	(yrs)		(mg./24hr)	(μg./100ml.)	(%)	(μg./100ml.)	(%)	(μg./100ml.)
				1. Normal group				
3. W.J.	35	M	249.0	63.3	39.5	25.0		
4. W.H.	55	M	216.0	68.2	32.7	22.3		
5. M.L.	42	F	225.0	27.6	25.7	7.1	24.3	6.7
6. M.H.	35	F	218.0	43.7	40.0	17.5	28.5	12.5
7. M.R.	23	F	268.0	60.1	45.5	27.3	44.4	26.7
Range	23-55			27.6-68.2	25.7-45.5	7.1-27.3	24.3-44.4	6.7-26.7
Mean	38			52.6	36.7	19.8	32.4	15.3
S.D.				16.73	7.63	8.01	10.67	10.29
S.E.				7.48	3.40	3.58	6.10	5.94
				3. 'Cushingoid' group				
18. A.D.	19	F	358.0	128.1	58.1	74.4		
19. D.M.	21	M	194.0	73.3	37.1	27.2		
23. D.S.	34	F	233.0	63.0	37.5	23.6		
24. M.K.	15	F	241.0	38.8	19.4	7.5	13.5	5.2
Range	15-34			38.8-128.1	19.4-58.1	7.5-74.4		
Mean	22.5			75.8	38.0	33.2		
S.D.				11.93	15.81	28.77		
S.E.				5.96	7.90	14.38		
				4. Obese group				
28. T.A.	17	M	199.0	53.6	38.3	20.5		
31. A.I.	24	F	199.0	68.5	40.0	27.4		
Range	17-24			53.6-68.5	38.3-40.0	20.5-27.4		
Mean	20.5			61.1	39.2	23.9		
S.D.				9.00	1.20	4.89		
S.E.				6.36	0.85	3.46		

Table (21)
Plasma level and plasma-protein binding of cortisol
increase after ACTH

Case	Total plasma cortisol: increase		% NPB cortisol (in vitro): increase		NPB cortisol (in vitro): increase	
	(μg.)	(%)	absolute	(%)	(μg.)	(%)
1. Normal group						
3. W.J.	53.6	552	25.4	180	23.6	1724
4. W.H.	54.8	408	16.7	104	20.1	937
5. M.L.	18.3	196	10.9	73	5.7	414
6. M.H.	35.8	453	27.0	207	16.5	1650
7. M.R.	44.7	290	30.0	193	24.9	1042
Range	18.3-54.8	196-552	10.9-30.0	73-207	5.7-24.9	414-1724
Mean	41.4	380	22.0	151	18.2	1153
S.D.	15.0	139	7.9	59	7.6	542
S.E.	6.7	62	3.5	26	3.4	242
3. 'Cushingoid' group						
18. A.D.	112.9	742	35.1	152	70.9	2025
19. D.M.	43.7	147	19.6	112	22.5	478
23. D.S.	52.4	494	22.1	143	21.9	1356
24. M.K.	24.5	171	9.2	90	6.0	413
Range	24.5-112.9	147-742	9.2-35.1	90-152	6.0-70.9	413-2025
Mean	58.4	388	21.5	124	30.4	1068
S.D.	38.1	284	11.5	28	28.9	769
S.E.	19.0	142	5.7	14	14.4	384
4. Obese group						
28. T.A.	47.2	737	13.9	56	18.9	1222
31. A.I.	51.7	307	27.5	220	25.3	1204
Range	47.2-51.7	307-737	13.9-27.5	56-220	18.9-25.3	1204-1222
Mean	49.4	522	20.7	138	22.1	1213
S.D.	3.0	303	9.6	115	4.4	12
S.E.	2.1	214	6.8	57	3.1	8

significant separation of cases of Cushing's syndrome from either normal or obese subjects by employing ACTH stimulation followed by total plasma cortisol estimations. Thus, Dyrenfurth et al (1960) obtained a mean of 62.1 µg. cortisol/ 100 ml. plasma in cases of Cushing's syndrome against 35.5 µg./100 ml. and 37.8 µg./100 ml. for normal males and females respectively. Hoet et al (1961) obtained the following means on the first day of ACTH stimulation 42.2 ± 2.0 (S.E.) µg./100 ml., 44.8 ± 8.7 µg./100 ml. and a range of 79.1 - 108.2 µg./100 ml. for normal and obese subjects and cases of Cushing's syndrome respectively and 53.7 ± 7.7 µg./100 ml., 55.1 ± 18.0 µg./100 ml. and a range of 69.9 - 107.0 µg./100 ml. for the same groups on the second day of ACTH stimulation. Beck et al (1962) obtained mean values of 35.5 µg./100 ml, 37.8 µg./100 ml. and 62.1 µg./100 ml. for normal males, normal females and patients with Cushing's syndrome respectively. Expressed increase of plasma cortisol over control levels, values were 21.2, 24.0 and 41.0 µg., but when expressed as percent increase over control values the values were about 170, 135 and 200% respectively; this supports the findings of the present study which show the superiority of expressing results as their absolute value and not as increased values over the control.

Grant (1963) believed that the ACTH test was the most important and reliable test to differentiate normal and obese subjects on the one hand from Cushing's syndrome on the other, employing plasma cortisol estimations. He reported levels of 30.0 - 55.0 µg. cortisol/100 ml. plasma in normal subjects and found that obese subjects gave the same or slightly higher levels but that the increase in them always parallelled that of the normal subjects, whereas cases of Cushing's syndrome gave levels well above the normal after an/

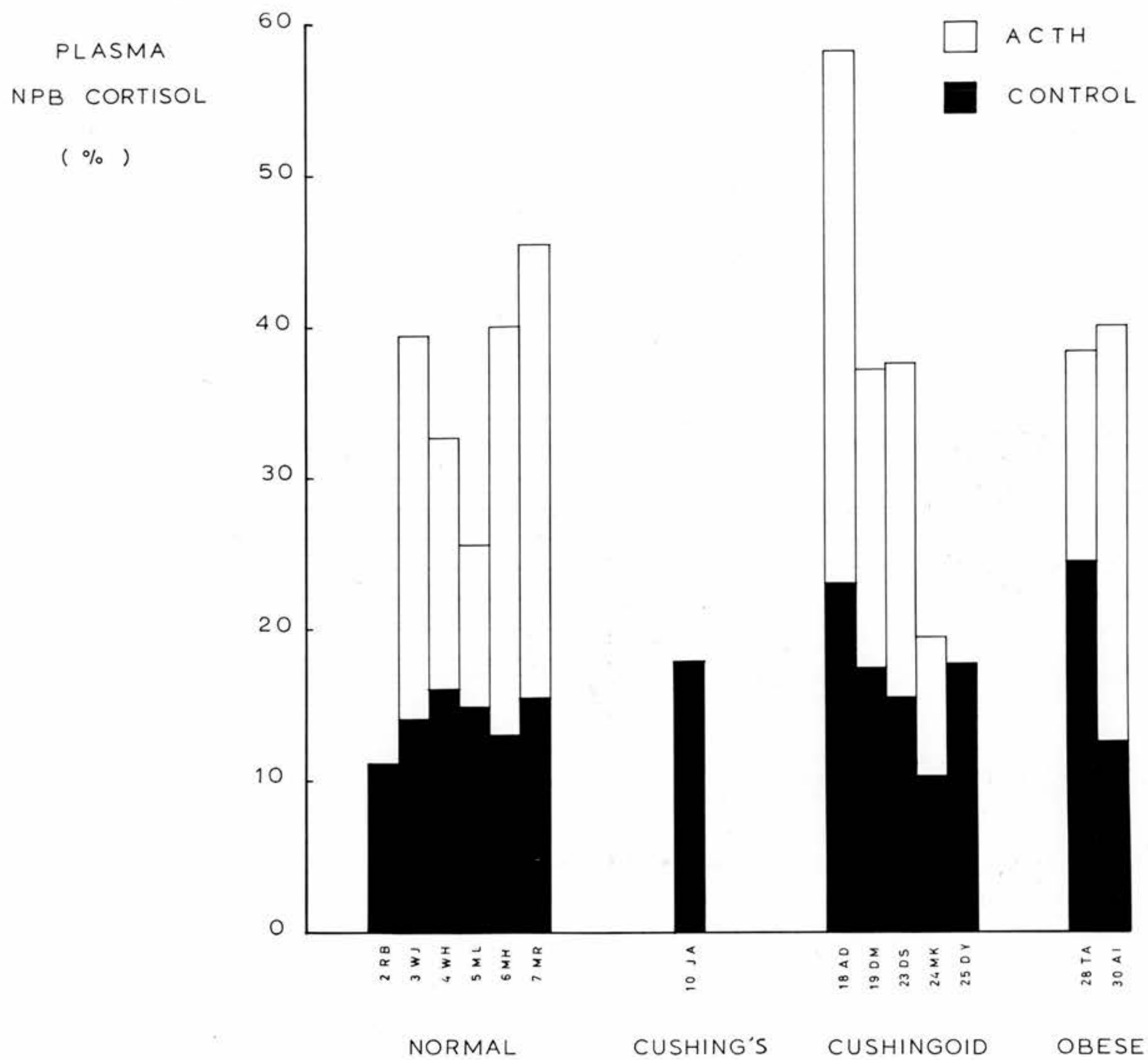


FIGURE 25 : % PLASMA NON-PROTEIN-BOUND CORTISOL

CONTROL ESTIMATIONS AND ESTIMATIONS AFTER ACTH

an ACTH infusion lasting 4 hours. Jailer (1962) reported that 45 of his 46 patients with Cushing's syndrome due to bilateral adrenal hyperplasia showed hypersensitivity to ACTH; in one case there was a normal response but this rose above normal after 3 days stimulation by ACTH.

(b) Non-protein-bound cortisol

i- Percent non-protein-bound cortisol. The highest level was reached by a patient in the 'Cushingoid' group, but overlapping was again observed, and there was no significant difference between the means of the three groups. Comparing the means in each group to the corresponding control values revealed a highly significant increase in the normal group and a significant increase in the 'Cushingoid' group after ACTH stimulation but no significant difference in the obese group.

Expressing the results of this parameter as "absolute increase above control value" the highest value was observed in one of the 'Cushingoid' patients, but the ranges overlapped in the three groups and there was no significant difference between the means of any two of them. When results were expressed as "percent increase above control value", there was even greater overlap, and no significant difference between the means.

ii- Non-protein-bound cortisol expressed as its absolute value (in µg./100ml). The highest level was reached by one of the 'Cushingoid' patients, but there was no significant difference between the means of any two groups, although the increase of the mean in the normal and in the obese groups over their corresponding control values were highly significant, and for the 'Cushingoid' group the increase was significant.

Expressing the results of this parameter as "increase over the control level" in µg., the highest level was again observed in one of the 'Cushingoid' patients. Overlap/

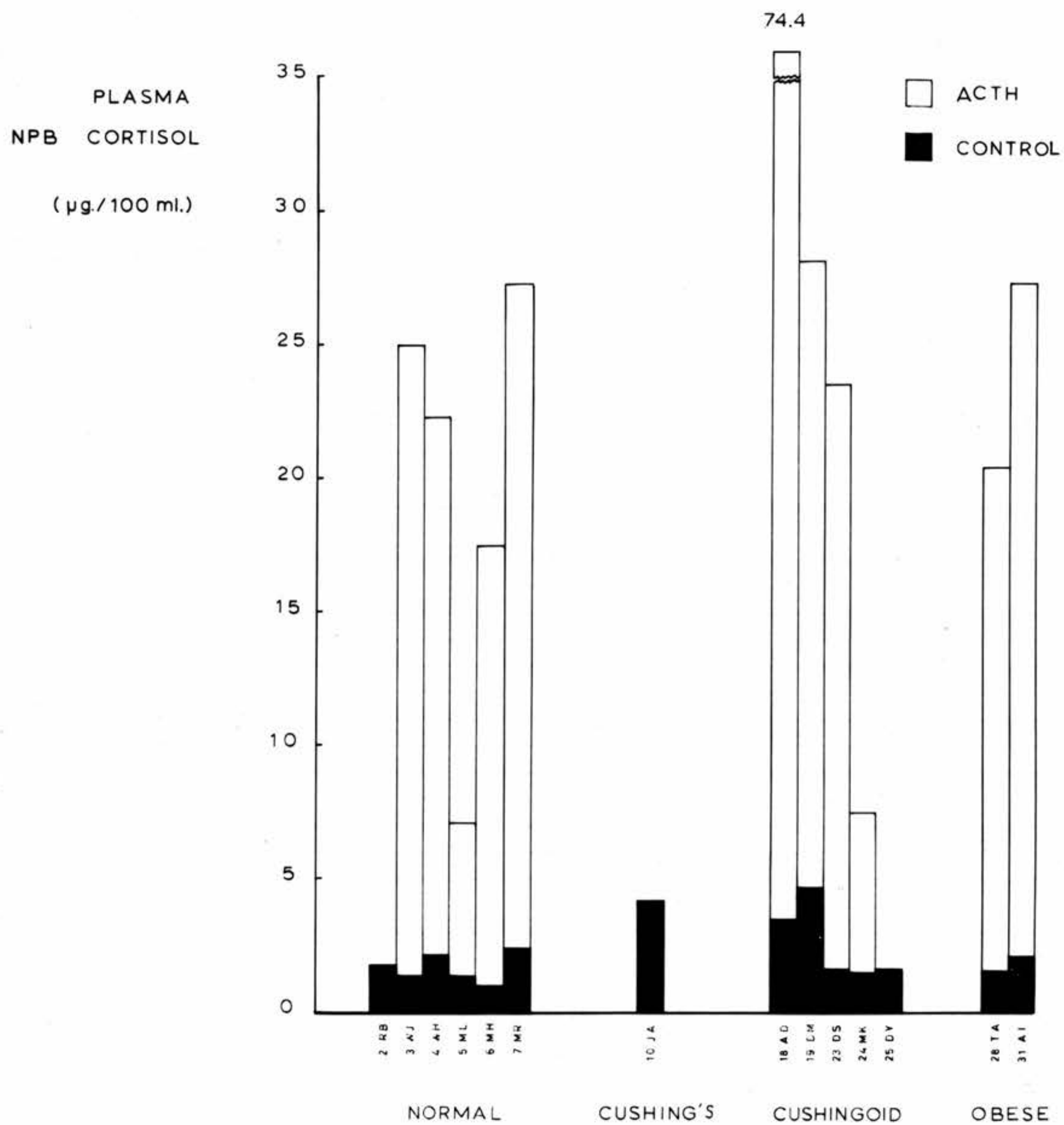


FIGURE 26 : PLASMA NON - PROTEIN-BOUND CORTISOL

CONTROL ESTIMATIONS AND ESTIMATIONS AFTER ACTH

Overlap occurred among the rest of the levels and there were no significant differences between the means in the three groups. Expressing results as "percent increase over the control value" also showed marked overlapping of the ranges with no significant differences between the means.

In a study of plasma cortisol in 7 normal subjects before and after a standard intravenous ACTH test, Doe et al (1960 b) reported that a rise occurred only in the NPB fraction, the range of this rise being 20.0 - 36.0 µg. NPB cortisol/100 ml. plasma (mean 27). The same group of workers in 1963 reported a normal mean of 13.2 µg./100 ml. in normal subjects, and a level of 41.7 µg./100 ml. in a patient suffering from Cushing's syndrome.

Non-protein-bound cortisol as estimated by the in vivo method

When NPB cortisol was expressed as its absolute value (µg./100 ml.) or as a percent of total plasma cortisol, the difference between the level of the 'Cushingoid' patient and the mean of the three normal subjects was not significant. The levels here are lower than the corresponding *in vitro* figures, the difference being sometimes quite marked. Provided both techniques were performed under exactly the same conditions, and the amount of radioactive cortisol added to the plasma in the *in vitro* method was of the same order as that present in the plasma in the *in vivo* method, the only explanation for the discrepancy between these two techniques must be that more binding occurs inside the human body than in the test tube.

3. Estimations after dexamethasone suppression

As stated previously, CPR after dexamethasone suppression could not be estimated by the blood method, because of the extremely low plasma levels. Similarly, the radioactivity of the ultrafiltrate gave figures almost as low as the background. Accordingly, neither plasma cortisol nor the NPB cortisol level could be calculated. This applied to all cases to whom dexamethasone was administered except J.A. (Case 10), a patient with Cushing's syndrome.

The test was applied to (1) case 10. (J.A.),one of the patients with Cushing's syndrome, (2) patients 18. (A.D.), 20. (D.M.), and 23. (D.S.) of the 'Cushingoid' group, and (3) patients 28. (T.A.) and 31. (A.I.) of the obese group. In patient J.A., suppression was not marked and a CPR of 33.6 mg./24 hrs., plasma cortisol of 9.0 μg./100 ml. and an NPB cortisol of 7.5% (0.67 μg./100 ml.) were observed. The experiment showed that, at least in some obese and 'Cushingoid' patients, marked suppression of adreno-cortical activity occurred after dexamethasone. More data are needed, especially in Cushing's syndrome and in obese individuals to see if this test is of value in differentiating these two conditions.

The dose of radioactive cortisol (2 μc.) contains between 30 and 35 μg. of cortisol. When introduced into the body it increased the cortisol level by less than 0.3 μg./100 ml. Introduction of a bigger dose to facilitate counting the ultrafiltrate, i.e. obtaining readings well above the background, could significantly raise the plasma level of cortisol and possibly affect the degree of binding, thus rendering interpretation of the results dubious.

Grant (1963), however, believed that variable results were obtained in the dexamethasone test. He stated that normal (maximal) suppression of plasma cortisol/

cortisol usually occurred with the smaller dose (0.5 mg. 6 hourly for 2 days), suppression of cases of adrenal hyperplasia occurred with the larger dose (2 mg. 6 hourly for 2 days), and that no suppression occurred when either dose was given to patients with autonomous tumours. He noticed, however, that cases were sometimes encountered in whom a response occurred that was intermediate between hyperplasia and autonomous tumours, and he suggested that these cases could be due to nodular hyperplasia. He stated, however, that response to dexamethasone did not always correlate with definite pathology of the adrenal cortex.

Conclusion

The simultaneous estimation of CPR, plasma cortisol and plasma NPB cortisol offers the following advantages:-

1. Three of the most accurate tests for the assessment of adrenocortical function are carried out simultaneously. The accuracy of these tests depends ultimately on the accuracy of the fluorometer and/or the radioactive counting machine. The accuracy (99% confidence limit) of the fluorometer was found to be 5.5% for samples containing 0.1 μg. cortisol (≡ 10 μg./100 ml. plasma) while that of the counting machine ranged from less than 1% for highly active specimens to 10% for specimens of low activity.
2. The results can be obtained after three days' work.
3. The use of radioisotopic cortisol to correct for losses during the procedure makes the estimation of plasma cortisol extremely accurate. The ranges given should therefore represent the "actual ranges" and not just "the ranges for the method".

4./

4. Because of the great sensitivity of counting equipment, a relatively small amount of plasma is needed for estimation of NPB cortisol by ultrafiltration, viz 5 ml; other methods, using chemical techniques for estimation of cortisol in the ultrafiltrate, need at least 15 ml. of plasma.

5. NPB cortisol is estimated 'directly'. This is in contrast to all other methods (except the ultrafiltration methods), which either estimate the CBG (or transcortin), or its 'capacity' to combine with cortisol. These methods, besides being 'indirect' methods for the assessment of the NPB cortisol, are usually time-consuming since a few days are needed for the equilibrium dialysis to be completed. These methods have, therefore, to be carried out at abnormally low temperatures (usually 4°C), to prevent bacterial growth, which could reduce the cortisol in the plasma and accordingly affect the accuracy of the results. In the present study, ultrafiltration for 5 hours was enough to obtain an ultrafiltrate containing a measurable amount of radioactivity, and ultrafiltration could be carried out at 37°C. The present technique would appear, therefore, to be more accurate from the physiological point of view, since it yields results which should correspond very closely to the conditions inside the human body.

6. The main reason for using radio-isotopic techniques is to introduce into the body an amount of cortisol that is too little to cause a significant increase in the plasma level of cortisol or in its degree of binding by plasma proteins, but which, at the same time, contains an amount of radioactivity easily detectable in the final extracts by sensitive counting equipment. At first, it/

it was not possible to produce cortisol of a high specific activity. Thus Peterson and Wyngaarden (1956), the originators of this method, used radioactive cortisol doses containing 200 - 500 µg. cortisol. When radioactive cortisol of higher specific activity became available, it was possible to introduce into the body smaller amounts of cortisol. Thus, Hoet el al (1961), for instance, used doses of radioactive cortisol containing between 60 and 80 µg., and Chen et al (1961) used radioactive cortisol of S.A. 4.17 µc./mg. In the present research, cortisol of a very high S.A., viz 60 µc./mg., was used, which meant that the amount of cortisol introduced into the body (about 30 µg.) was much less likely to affect either the cortisol level or degree of binding to plasma proteins. Results obtained with the more highly radioactive cortisol should more closely represent the conditions inside the body.

7. Lastly, an *in vivo* modification for the estimation of NPB cortisol has been described. In this technique equilibration between radioactive and non-radioactive plasma cortisol was allowed to take place under physiological conditions, in the body itself. In the *in vitro* method, the temperature and pH of the plasma during the experiment were adjusted to simulate conditions in the human body, but, in spite of this, differences were noticed between the results of the two methods. This showed that, even when temperature and pH were adjusted, equilibration of radioactive cortisol inside the human body was more efficient than in the test tube, since the degree of binding was always more in the *in vivo* method.

The factor, or factors, responsible for this difference in binding between the two methods is not known. It may be that one of the tissues in the body is necessary/

necessary to enhance the union of cortisol to its binding sites on CBG. The results obtained by the *in vivo* technique should, therefore, be more representative of the physico-chemical processes which occur inside the human body, and should at present be regarded as having greater physiological value.

The *in vitro* method gave a higher value for the patient with Cushing's syndrome than the upper limit of the range for the normal group. This suggests that it could be an extremely important index for assessing adrenocortical function, yet the ranges given could be the ranges of the method rather than physiological ranges. The author, therefore, recommends using the *in vivo* method, the results of which should represent both the ranges for the method and the actual ranges, thus reproducing the conditions inside the human body.

C. Treatment of Cushing's syndrome by adrenalectomy

1. Unilateral adrenalectomy

Assessment of adrenocortical function in a patient with Cushing's syndrome (case 10., J.A.) after unilateral adrenalectomy was performed by repeating the tests of cortisol production rate and urinary steroid excretion after the operation. Table 22 provides the results of these estimations, and allows comparison to be made between pre-operative and post-operative values.

Table (22)

Adrenocortical function after unilateral adrenalectomy

case 10 (J.A.)

Test		Control	After unilateral adrenalectomy*
a. CPR: blood method			
Miscible pool	(mg.)	7.47	4.4
$t_{\frac{1}{2}}$	(min.)	42	87.0
Rate of turnover	(pool/hr)	0.99	0.5
Production rate	(mg./24 hrs.)	177.6	52.8
b. CPR: urine method			
Dose excretion first 24 hr.	(%)	80.0	44.7
S.A. THE	(cpm/μg.)	5.61	8.92
Production rate	(mg./24 hrs.)	107.0	45.5
c. 17-KS	(mg./24 hrs.)	18.7	4.8
d. Total 17-KGS	(mg./24 hrs.)	54.9	20.3

* Tests performed 3 weeks after the operation

The miscible pool and rate of turnover dropped to nearly half their original value, and the production rate by the blood method dropped from 177.6/

177.6 to 52.8 mg./24 hrs. The latter value was, however, still higher than normal, and the size of the miscible pool was still above normal. Cortisol production rate by the urine method showed a similar drop, but the new level was still well above normal. On the other hand, 17-KS excretion fell from a level just above the normal range to a below-normal level, and total 17-KGS, which were originally moderately elevated, fell to the upper level of normal. This shows again the superiority and sensitivity of cortisol production rate tests over measures of steroid excretion, especially 17-KS determinations, in detecting moderately elevated adrenocortical activity.

Hoet et al (1961) studied the CPR (blood method) in two patients suffering from Cushing's syndrome after unilateral adrenalectomy. They reported that the miscible pools were 0.65 and 3.0 mg., the rate of turnover 0.59 and 0.47 pool/hr. and CPR 0.38 and 1.4 mg./hr. Liddle (1960) found that patients suffering from Cushing's syndrome due to adrenal hyperplasia showed resistance to endogenous-ACTH suppression by dexamethasone post-operatively indistinguishable from that observed before operation. This agrees with the findings of the present study (see table 14).

Signs and symptoms of Cushing's syndrome in patient J.A. were still present, after unilateral adrenalectomy, although considerably improved. Liddle (1960) on the contrary, stated that unilateral adrenalectomy did not result in a significant decrease in basal steroid excretion and failed to produce any clinical improvement. The persistence of the clinical manifestations of Cushing's syndrome in patient J.A., with high CPR and urinary steroid excretion after unilateral adrenalectomy, justified a second stage adrenalectomy and maintenance therapy.

2./

2. Bilateral adrenalectomy with adrenocortical auto-transplantation

As stated in the introductory discussion of Cushing's syndrome (Chapter III), the treatment of choice has been adrenalectomy (total, subtotal or unilateral). Sometimes hypophysectomy or irradiation of the pituitary gland may be employed, or, in desperate cases, adrenocortical inhibitory therapy, although this is still in its experimental stage. It was also mentioned then that any of these lines of treatment suffered disadvantages such as recurrence of Cushing's syndrome or the administration of maintenance therapy throughout life.

A new approach was the introduction of the auto-transplantation operations, as a method of surgical treatment which did not suffer from the above drawbacks. The principle of such operations is that bilateral adrenalectomy is performed, part of one adrenal gland is divided into small pieces, and these are transplanted, in small "heaps", into the rectus sheath of the anterior abdominal wall. The two important advantages offered by this type of operation are: (1) part of the adrenal tissue is left inside the body and this can secrete the necessary amounts of corticosteroids, thus relieving patients from the requirement of being maintained on steroid therapy throughout the rest of their lives; (2) the transplanted adrenal fragments are located in an easily accessible part of the patient's body, so that further removal of adrenal tissue can be easily performed, even under local anaesthesia, if higher-than-normal steroids are still being secreted.

Table (23)/

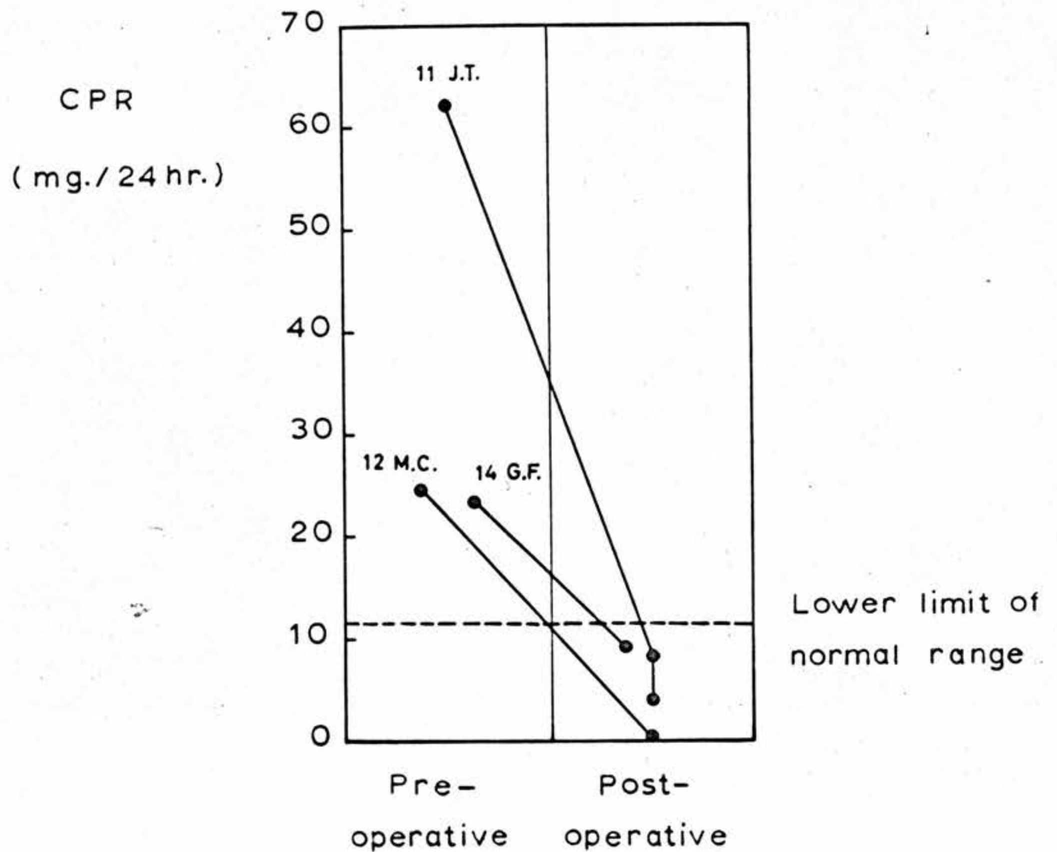


FIGURE 27: CORTISOL PRODUCTION RATE (URINE METHOD) BEFORE AND AFTER BILATERAL ADRENALECTOMY WITH ADRENOCORTICAL AUTO-TRANSPLANTATION

Table (23)

Adrenocortical function after bilateral adrenalectomy and auto-transplantation

Case	CPR Urine method (mg./24hrs)	17-KS (mg./24hrs)	Total 17-KGS (mg./24 hrs)
11. J.T. pre-operative	62.2	14.2	15.8
post-operative (2 months)	8.3	1.6	3.0
(7½ months)	4.2	1.6	3.7
12. M.C. pre-operative	24.6	10.2	21.8
post-operative (2½ months)	0.44	2.6	2.4
14. G.F. pre-operative	23.0	32.7	45.4
post-operative (1 year)	9.0	11.5	18.4 (gl.)*

*glycosuria

Table 23 summarises the results of cortisol production rate and steroid excretion tests before and after such an operation in three patients with Cushing's syndrome (see also figure 27). In the first case (11., J.T.) a production rate of 8.3 mg./24 hrs. was detected two months after the operation, associated with marked improvement of the clinical condition. Seven-and-a-half months after operation, however, the patient started to show signs and symptoms of hypoadrenocorticism; cortisol production rate was estimated, found to be 4.2 mg./24 hrs., and steroid maintenance therapy had to be started. In the second case, (12., M.C.), a post-operative cortisol production rate of a negligible value was detected two-and-a-half months after the operation, associated with a clinical picture of hypoadrenocorticism; again, steroid maintenance therapy had to be started. In the third case (14., G.F.), one year after operation a cortisol/

cortisol production rate just below the lower limit of normal was detected, associated with a marked clinical improvement of the patient's condition, and it was not considered necessary to give any replacement therapy. Glycosuria, however, persisted in this third patient after operation, which suggests that, at least in some cases, glycosuria or even Cushing's syndrome itself are caused by pituitary or hypothalamic disorder rather than by an adrenal disorder.

Only one sixth of the total adrenal tissue was transplanted in these three cases. This should, theoretically, have given a lower than normal secretion of cortisol in all of them, and the ultimate failure of two out of these three operations could therefore be due to the surgeon having transplanted an amount of adrenal tissue that was insufficient to secrete the normal requirements of steroids. This small amount of transplanted tissue, perhaps after temporary hypertrophy, probably underwent exhaustion atrophy. These suggested changes in the adrenocortical tissue were described a long time ago by Selye (1946), who defined three consecutive stages in the "stress or adaptation syndrome", namely alarm, resistance and exhaustion. The second of these phases has been associated with hypertrophy and hyper-reactivity of the adrenal cortex, and the third phase only occurs if the degree or persistence of stress exhausts the adrenal defence mechanism. Leaving too little adrenal tissue inside the body to deal with the normal and stressful conditions of every-day life could well be such a stress to this small transplanted portion of adrenal tissue as to lead to the above mentioned changes.

Franksson et al (1956) were the first to suggest the auto-transplantation technique./

technique. They later on (Franksson et al, 1959) described 5 females, suffering from Cushing's syndrome, who had been treated by this method. The post-operative period ranged between 3 months and 2 years. Symptoms subsided in all cases and little or no exogenous steroids were required post-operatively. The adrenal pathology in these 5 cases was diffuse adrenal hyperplasia. These workers stated that it was very difficult to gauge correctly how much adrenocortical tissue to transplant, mainly because the cells of different adrenal glands seem to vary in steroid-producing capacity. The site of transplantation in their cases was the sartorius muscle. In the cases reported in the present study, the site chosen was the rectus sheath of the anterior abdominal wall, probably because it is a cleaner site than the thigh.

Conclusion

Because one of the three operations studied in this thesis was successful, the author believes that it is a potentially valuable method of treatment, and he suggests that the cortisol production rate estimation should be taken as the basis for deciding how much adrenocortical tissue to transplant. It is too early to state whether some other factors, related to the surgical technique itself, was responsible for the failure of some of these operations.

3./

3. Pathological examination of glands removed by adrenalectomy

Table 24 shows the results of pathological examination of adrenal glands removed from seven patients suffering from Cushing's syndrome. In only one case was the weight of the glands increased significantly above the normal limits; in this patient, the histology was normal. In two cases, adenoma was found (macroscopic or microadenomata), and signs of hyperplasia were detected in the rest of the glands.

The finding of histologically "normal" adrenal glands or adrenal glands of normal weight is not new. Sprague et al (1955) stated that 34 out of 88 individual adrenal glands removed from patients with Cushing's syndrome were histologically normal, that 50% of these glands weighed less than 8 grams and that 78% of them weighed less than 10 grams. Liddle (1960) reported that, after operation, 7 of his 27 cases had histologically normal glands, although diagnosed clinically and by 17-KGS and 17-KS estimations before and after dexamethasone as Cushing's syndrome with adrenal hyperplasia. Ekman et al (1961), similarly, reported that two of their 22 patients with Cushing's syndrome had normal glands. Symington and Jeffries (1962) also examined individual adrenal glands removed from patients suffering from Cushing's syndrome and found that 35% of them are histologically normal; 45% of them weighed less than 8 gram and 81% were less than 10 gram.

Patient J.A. (case 10), who showed clinical and laboratory findings typical of Cushing's syndrome, had histologically normal glands. This patient had a basal CPR about 6 times the normal value, and his adrenals were increased in weight to approximately twice the normal value. The aetiology of Cushing's syndrome in this patient could not be due to an "increased adrenal functioning mass", an idea previously suggested by Birke et al (1960). Incubation studies, using/

Table (24)

Examination of glands removed by adrenalectomy

Case	Weight of glands (gm) R	L	Naked eye appearance	Histological examination
10. J.A.	10	13		Normal histology. Histochemical studies using 4-C^{14}-progesterone showed a pattern similar to that obtained with most hyperplastic glands and different from that of normal or nodular hyperplastic glands or glands with tumours.
11. J.T.	fragmented	3.6	Normal	Broadening of the zona fasciculata. Clear cells are small. Appearance consistent with that of a gland which has been under ACTH stimulation.
12. M.C.	fragmented	6.76	Bilateral nodular hyperplasia	Each contained one relatively large adenoma .
13. J.M.	4.5	7.25	Normal	Outer half of cortex composed of cells rich in cytoplasmic lipid.
14. G.F.	fragmented		Normal	Very prominent zona fasciculata, moderately large 'clear' cells.
15. H.T.	fragmented		Normal	Zona fasciculata broader than normal, relatively numerous clear cells.
16. E.S.	fragmented		Normal	Hyperplasia with few micro-adenomata.

using radioactive cortisol precursors, of the adrenal glands of this patient revealed a hyperplastic pattern. It is possible that electron microscopy could detect changes, in such glands, that could not be detected by the ordinary histological techniques and this question remains to be answered.

D. Relationship of adrenocortical function to body weight, height and musculature

Table 25 represents the weight, height, surface area (calculated from the weight and height), total body lean cell mass and total body fat (calculated from the weight and total body water estimation), creatinine excretion (as a measure of body musculature), and cortisol production rate (blood method) in all cases of the four groups studied.

With the data on weight, the difference between the mean of the normal group and the three other groups is highly significant (see table 28). The increase above the desired weight in the obese group ranges from 4.1 - 117.9% (average 68.01).

In height, the difference between any two means is not significant.

In estimations of the surface area, the difference between the means of the normal group and the group of Cushing's syndrome is significant, and between the mean of the normal and obese group is highly significant.

With the total body lean cell mass, the mean of the normal group is significantly lower than that of either patients with Cushing's syndrome or patients in the 'Cushingoid' group. If the total body lean cell mass is expressed as "percent of the total body weight", the ~~same~~ opposite result is obtained.

If total body fat is expressed in Kg., the mean of the normal group is significantly lower than the obese group, and the difference between the normal group and both the group of Cushing's syndrome and the 'Cushingoid' group is highly significant. Expressed as "percent of the total body weight", the mean of the normal group is significantly lower than that of the group of Cushing's syndrome and the 'Cushingoid' group but not of the obese group.

The difference between the mean of the four groups, as far as creatinine excretion is concerned is not significant except between the normal and 'Cushingoid' group; 'Cushingoid' subjects excreted significantly increased amounts/

amounts of creatinine ~~than~~ over normal subjects. ~~This denotes that total muscle mass did not differ much among individuals of the four groups studied.~~

In 1956, Gray et al, studying the effect of weight reduction in obese subjects, suggested that in obese individuals with symptoms suggesting Cushing's syndrome, the possible effect of food intake on increased steroid excretion could be of diagnostic and therapeutic importance. Borth et al (1957) found that the differences in steroid excretion values for men and women was attributable entirely to the fact that, on the average, men are taller and heavier than women. Tanner et al (1959), studying healthy male medical students found a significant relationship between urinary 17-KGS excretion and weight, height and surface area. Significant relationships between weight and steroid excretion were also reported by Cohen (1958), Szenas and Pattee (1959), Simkin (1961) and Green et al (1961). Similar relations between weight and CPR were reported by Eckert et al (1961), Karl and Raith (1961) and Mlynaryk et al (1962).

Significant relationships between the surface area and steroid excretion (Poisnick and DiRaimondo, 1956; Tanner et al, 1959), surface area and CPR (Eckert et al, 1961) and between the surface area and the logarithm of the values for urinary excretion of major tetrahydro-α-ketolic metabolites of cortisol (Dohan et al, 1962) were reported.

Mlynaryk et al (1962) found that, when CPR was expressed as a function of creatinine excretion, the original raised CPR in obese subjects persisted, implying that muscle mass was not the factor involved in the higher CPR. These workers noticed that CPR, expressed as a function of weight, was similar

in/

in normal and obese groups, implying that the increased CPR in obese subjects was due to increased weight. These workers, therefore, concluded that the rise in CPR correlated better with body weight than with surface area, ideal body weight or estimated amount of fat. Similarly, DeMoor et al (1963) stated that the significant difference in hormone excretion between normal males and females disappeared when the data for hormone excretion were recalculated per unit body weight, height or surface area.

Schteingart et al (1963), on the other hand, found no correlation between either urinary 17-hydroxycorticosteroid excretion or CPR on the one hand, and the absolute body weight or the degree of overweight on the other. Similarly, in a group of normal and obese subjects, Gogate and Prunty (1963) found that the coefficient of correlation of CPR with body weight or surface area were not significant, viz 0.290 and 0.388 respectively.

As far as the relation between adrenocortical activity and body musculature is concerned, Romanoff et al (1961) found that, when CPR was expressed in terms of creatinine excretion, the difference between CPR of young and elderly men disappeared. Mlynaryk et al (1962), however, did not observe a relationship between adrenocortical activity and body musculature.

In view of these contradictory results, it was felt worthwhile to reassess the relationship between CPR and weight, height, surface area, lean cell mass, body fat and musculature in the groups studied in this research.

Assuming that cases of Cushing's syndrome, and probably some of the cases of the 'Cushingoid' group, represent abnormal and varying adrenocortical function, only the subjects of the normal and obese groups were used to investigate these relationships (see table 26 and figures 28-34). Patients of the Cushing's and 'Cushingoid' groups were, however, included in the diagrams for the sake of comparison.

Table(26)/

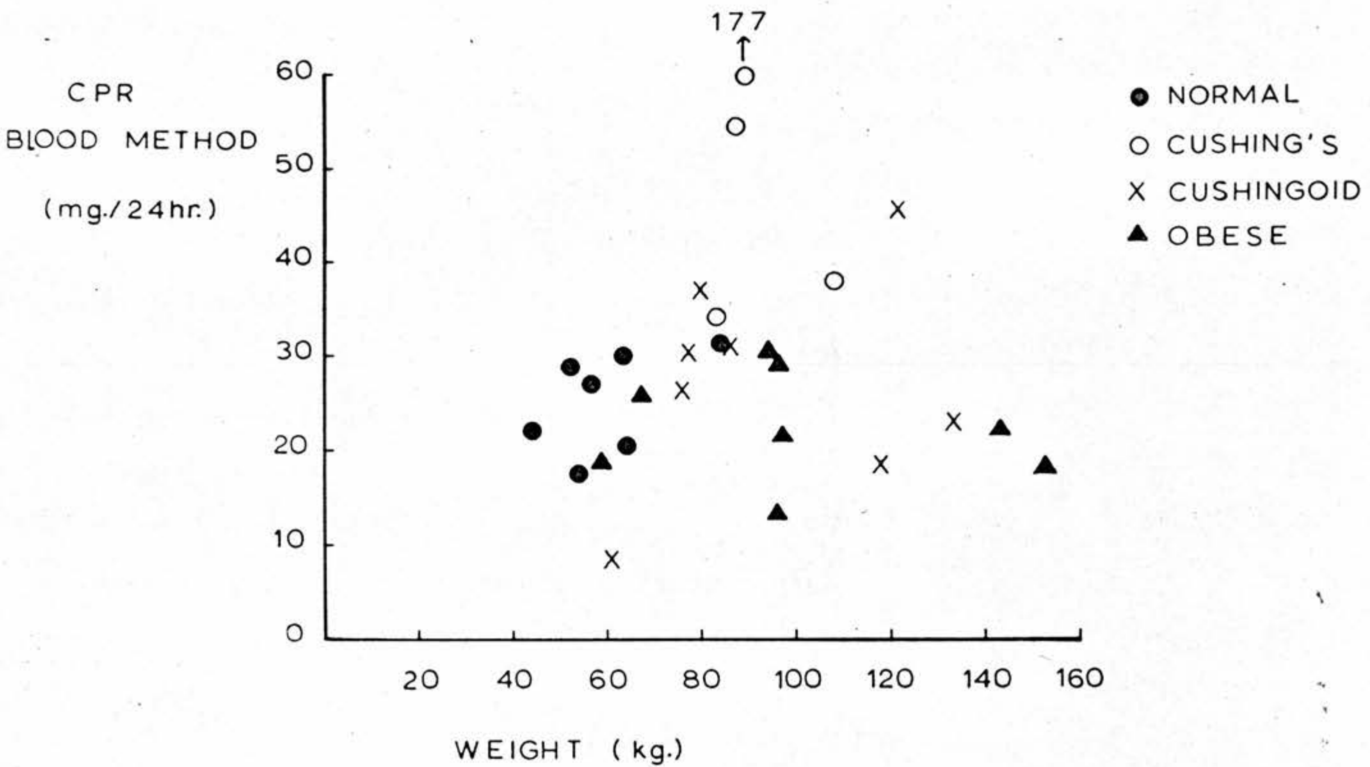


FIGURE 28 : RELATIONSHIP BETWEEN CORTISOL PRODUCTION RATE AND TOTAL BODY WEIGHT

FIGURE 29: RELATIONSHIP BETWEEN CORTISOL PRODUCTION RATE AND BODY HEIGHT

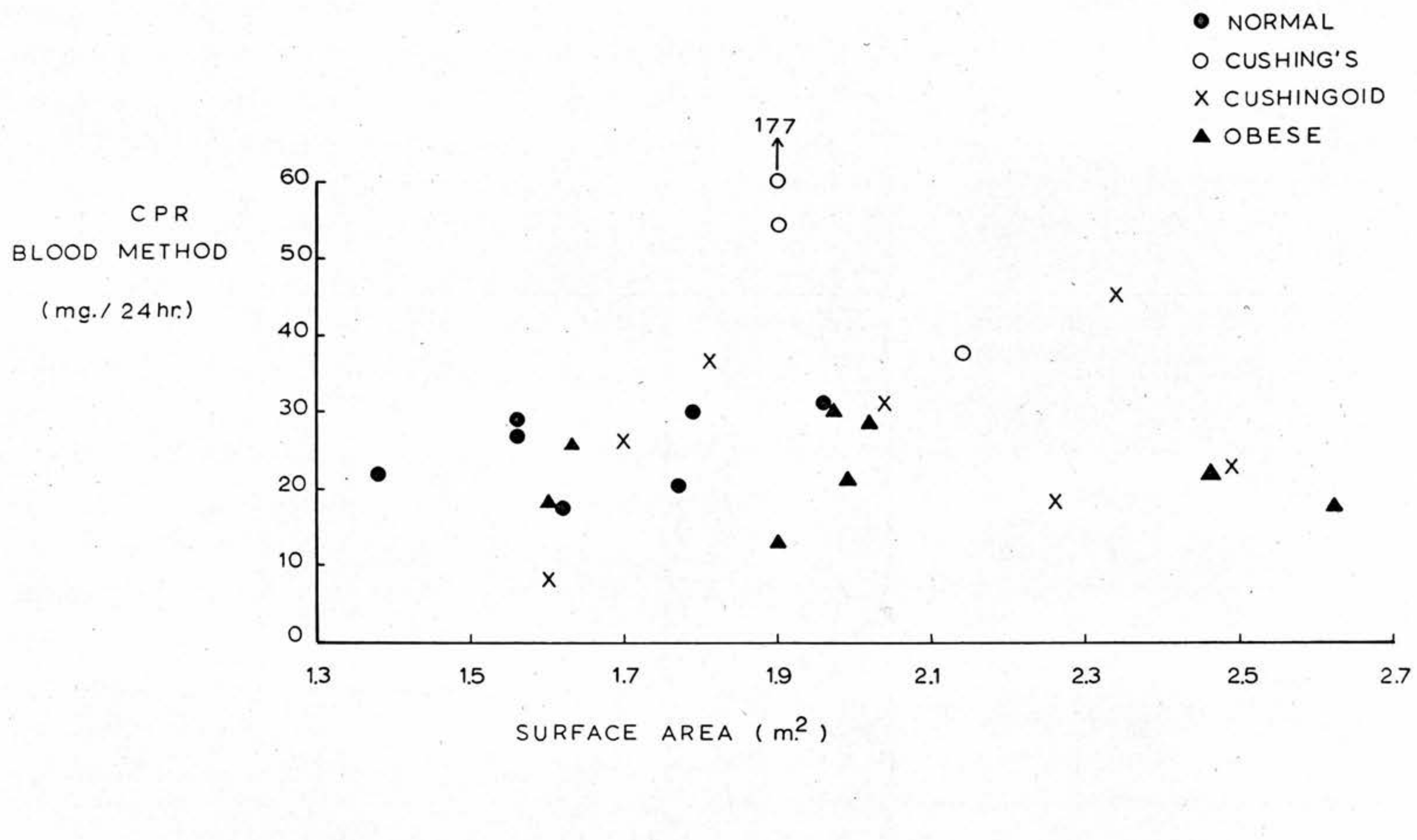


FIGURE 30: RELATIONSHIP BETWEEN CORTISOL PRODUCTION RATE AND BODY SURFACE AREA

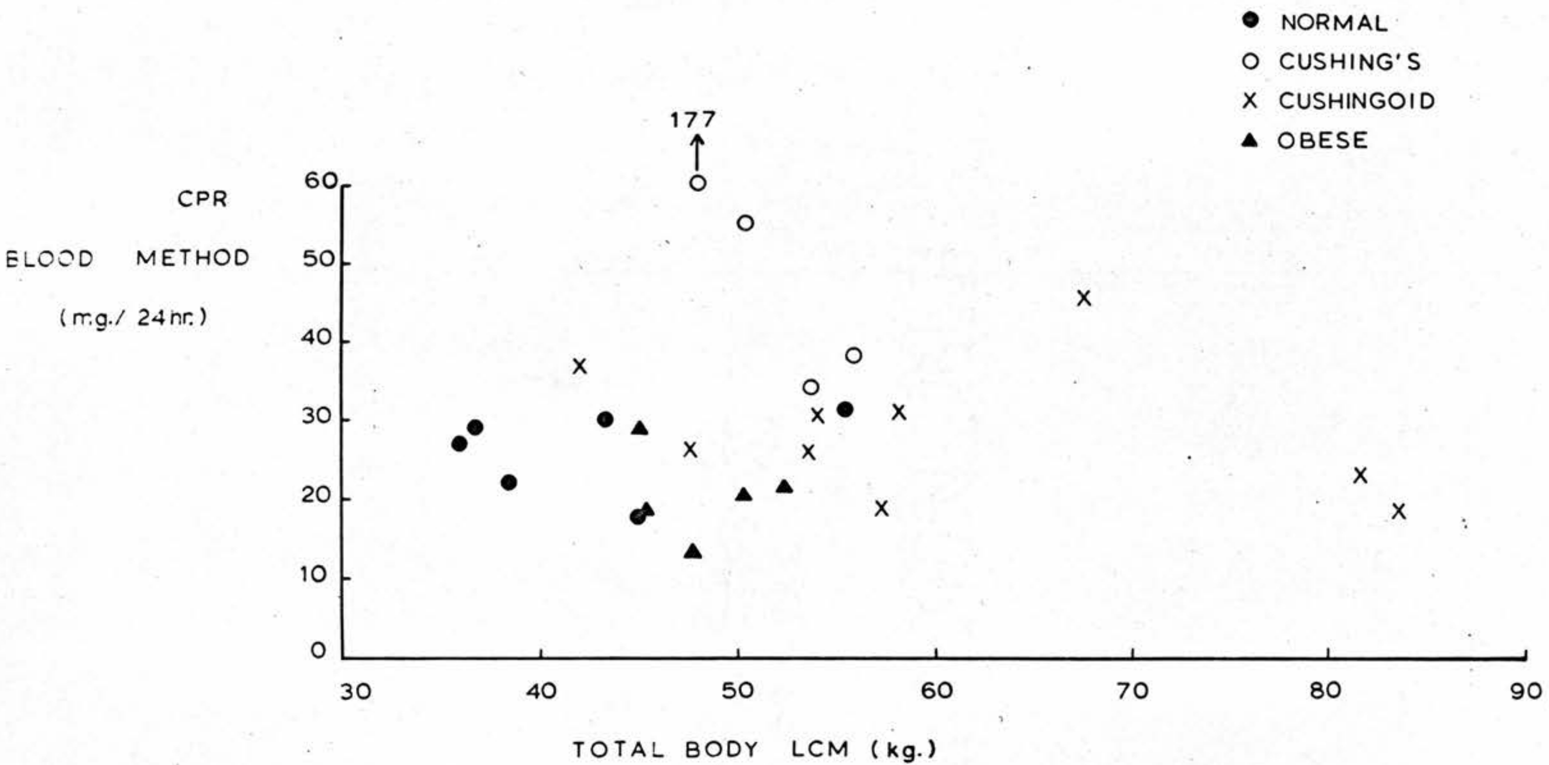


FIGURE 31: RELATIONSHIP BETWEEN CORTISOL PRODUCTION RATE AND TOTAL BODY LEAN CELL MASS

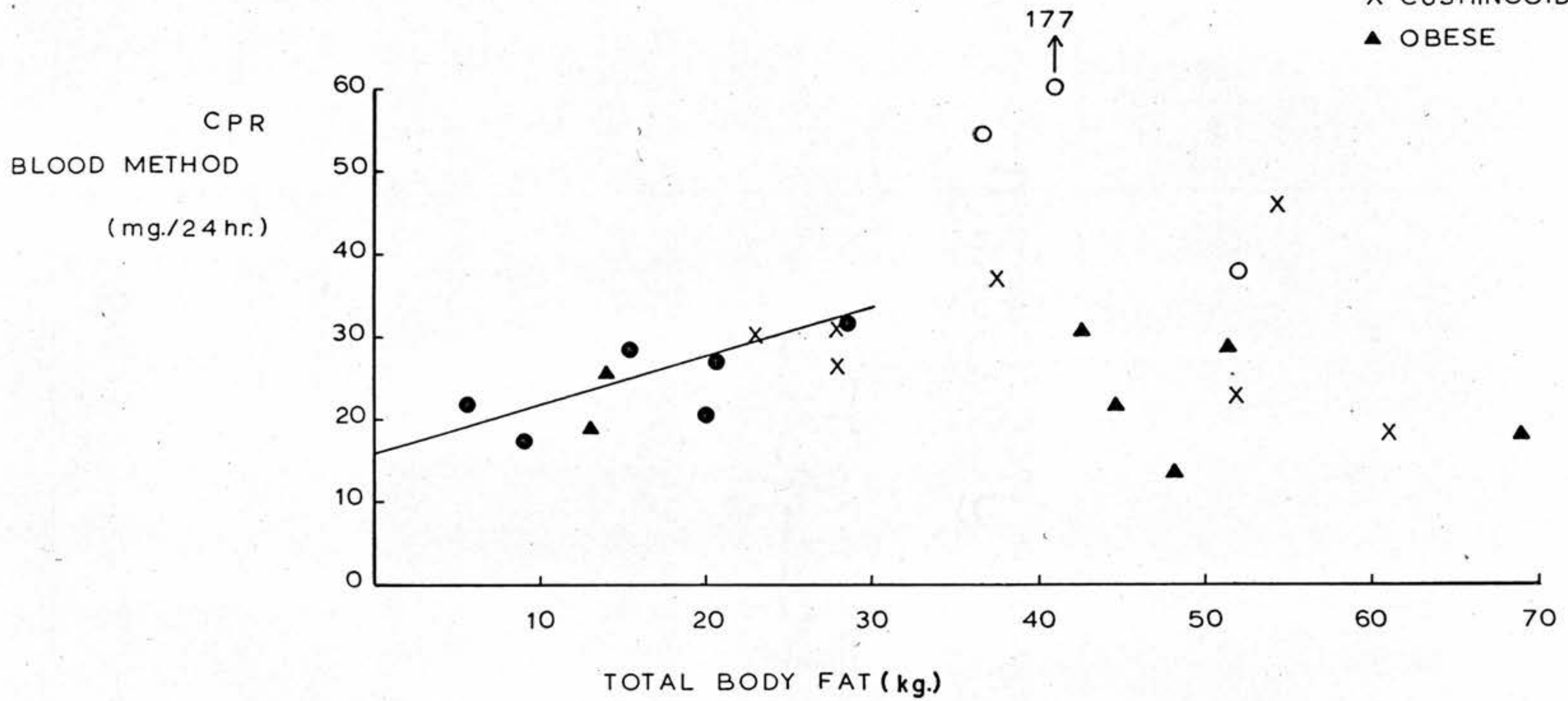


FIGURE 32: RELATIONSHIP BETWEEN CORTISOL PRODUCTION RATE AND TOTAL BODY FAT

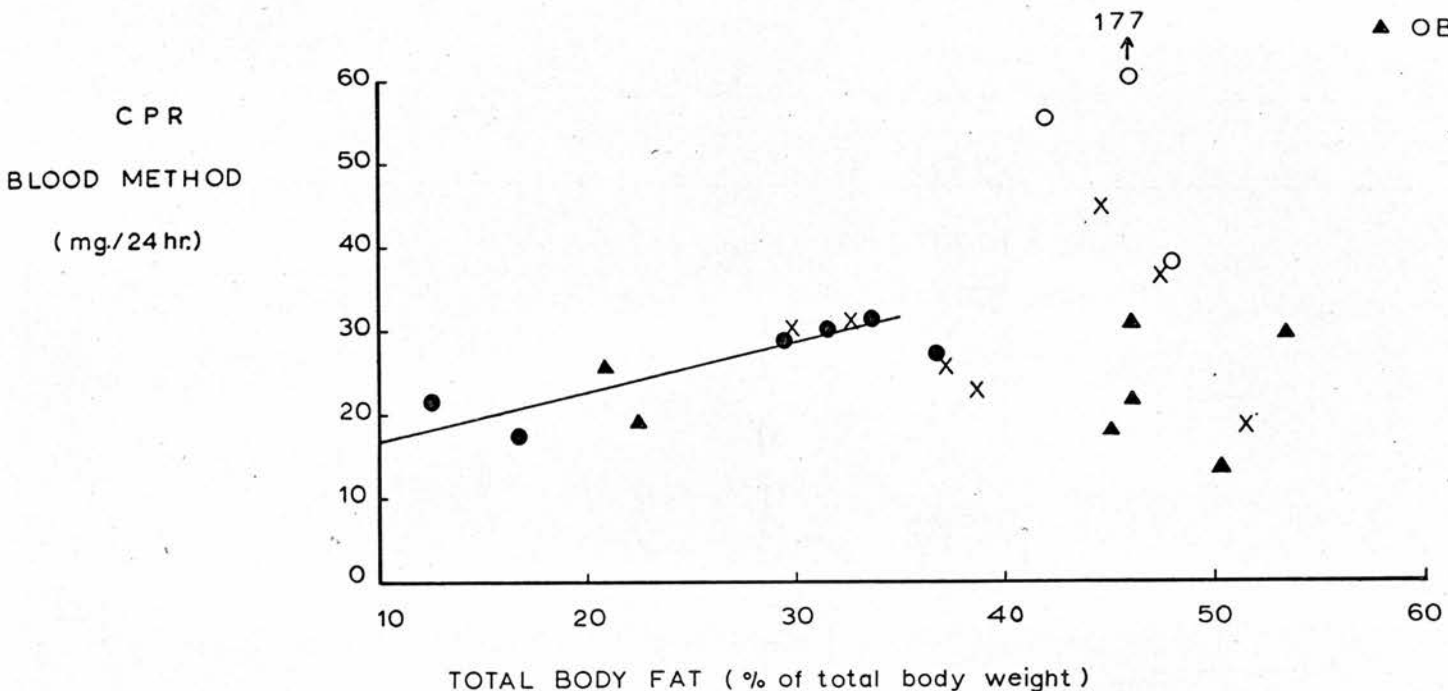


FIGURE 33 : RELATIONSHIP BETWEEN CORTISOL PRODUCTION RATE AND TOTAL BODY FAT AS A PERCENT OF TOTAL BODY WEIGHT

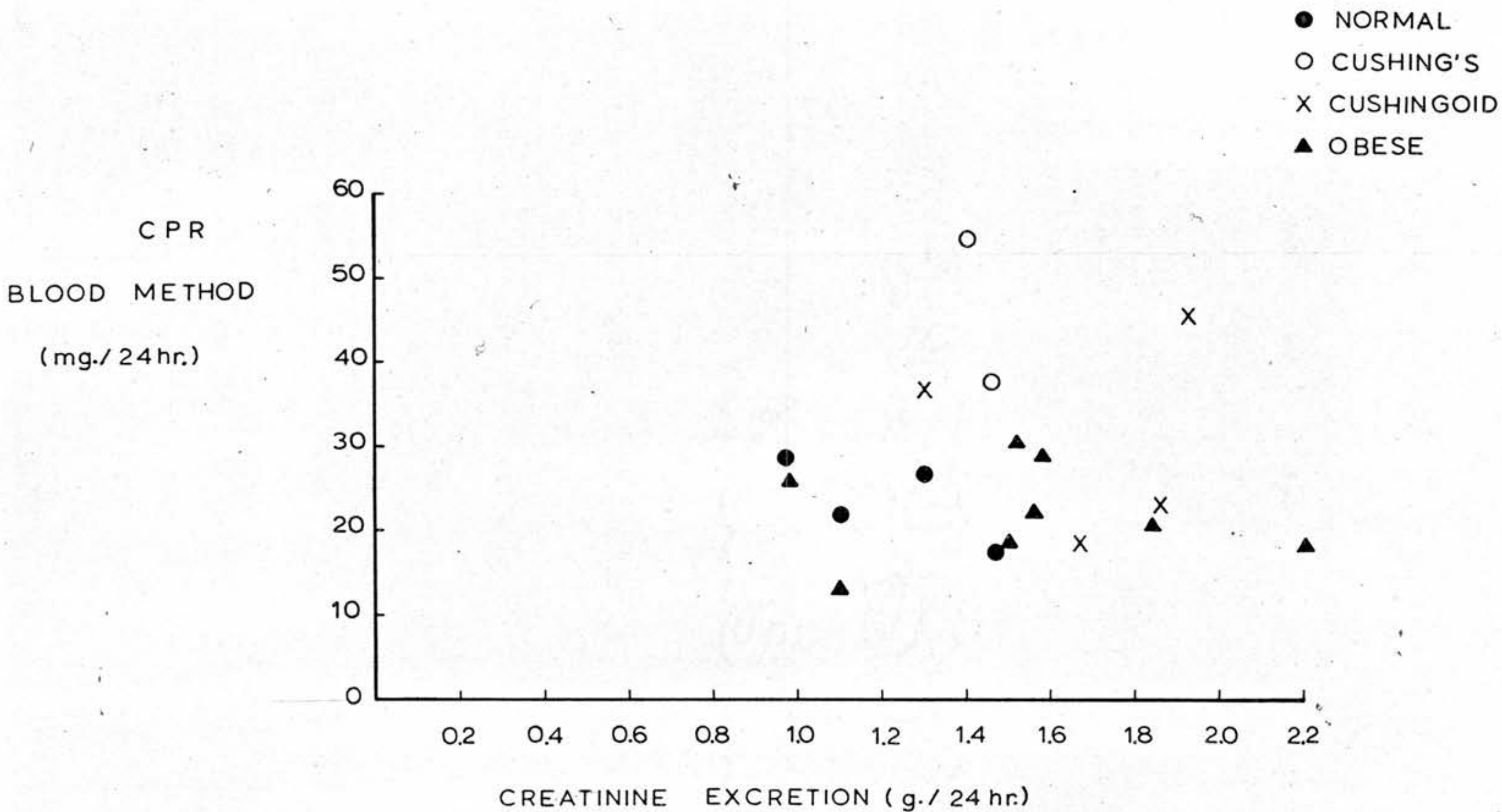


FIGURE 34: RELATIONSHIP BETWEEN CORTISOL PRODUCTION RATE AND CREATININE EXCRETION

Table (25)

Relationship between CPR and weight, height, surface area, body lean cell mass, body fat and body musculature.

Case	Age (yrs)	Sex	Weight (Kg)	Height (cm)	Surface area (m^2)	Total body water (l)	Total body LCM (Kg)	Total body LCM (% of total body weight)	Total body fat (KG)	Total body fat (% of total body weight)	Creatinine excretion (g/24hr)	CPR (control: blood method) (mg/24hr)
1. Normal group												
1. B.A.	29	M	63.0	175.0	1.77							20.4
2. R.B.	65	M	63.4	177.8	1.79	31.1	43.32	68.33	20.08	31.67		30.0
3. W.J.	35	M	54.0	170.0	1.62	32.3	44.98	83.30	9.02	16.70	1.47	17.5
4. W.H.	55	M	83.4	171.4	1.96	39.7	55.29	66.30	28.11	33.70		31.2
5. M.L.	42	F	44.0	156.2	1.38	27.6	38.43	87.35	5.57	12.65	1.10	21.8
6. M.H.	35	F	52.0	165.1	1.56	26.3	36.63	70.45	15.37	29.55	0.97	28.8
7. M.R.	23	F	56.7	157.4	1.56	25.8	35.93	63.37	20.77	36.63	1.30	26.9
range	23-65		44.0-83.4	156.2-177.8	1.38-1.96	25.8-39.7	35.93-55.29	63.37-87.35	5.57-28.11	12.65-36.63	0.97-1.47	17.5-31.2
mean	40.6		59.5	167.6	1.66		42.43	73.18	16.49	26.82	1.21	25.2
S.D.			12.47	8.347	0.19		7.27	9.77	8.31	9.77	0.220	5.3
S.E.			4.713	3.155	0.072		2.970	3.990	3.393	3.990	0.110	2.00
2. Cushing's group												
a. Operated upon												
10. J.A.	55	M	89.0	157.4	1.90	34.5	48.04	53.98	40.96	46.02		177.6
11. J.T.	17	F	87.0	160.0	1.90	36.2	50.42	57.95	36.58	42.05	1.40	54.7
12. M.C.	36	F	108.0	166.3	2.14	40.1	55.85	51.71	52.15	48.29	1.46	37.9
b. Not operated upon												
17. C.M.	18	F	83.3			38.6	53.76	64.54	29.54	35.46		34.1
range	17-55		83.3-108.0	157.4-166.3	1.90-2.14	34.5-40.1	48.04-55.85	51.71-64.54	29.54-52.15	35.46-48.29	1.40-1.46	34.1-177.6
mean	31.5		91.8	161.2	1.98		52.0	57.04	39.81	42.96	1.43	76.1
S.D.			11.04	4.57	0.139		1.11	5.62	9.48	5.62	0.046	68.3
S.E.			5.520	2.639	0.080		0.556	2.811	4.728	2.812	0.033	34.15
3. 'Cushingoid' group												
18. A.D.	19	F	79.8	157.4	1.81	30.1	41.92	52.53	37.88	47.47	1.30	37.0
19. W.C.	27	M	133.6	180.3	2.49	58.7	81.76	61.20	51.84	38.80	1.86	23.0
20. D.M.	21	M	121.8	175.2	2.34	48.5	67.55	55.46	54.25	44.54	1.93	45.6
21. T.M	18	M	86.3	177.8	2.04	41.7	58.10	67.30	28.20	32.70		31.2
22. C.A.	26	F	61.1	154.9	1.60							8.6
23. D.S.	34	F	118.1	170.0	2.26	41.1	57.24	48.47	60.86	51.53	1.67	18.7
24. M.K.	15	F	76.9	158.7	1.79	38.7	53.91	70.10	22.99	29.90		30.2
25. D.Y.	32	F	75.7	148.5	1.70	34.2	47.63	62.92	28.07	37.08	1.45	26.4
range	15-34		61.1-133.6	148.5-180.3	1.60-2.49	30.1-58.7	41.92-81.76	48.47-70.10	22.99-60.86	29.90-51.53	1.30-1.93	8.6-45.6
mean	24.0		94.2	165.4	2.00		58.30	59.71	40.58	40.29	1.64	27.6
S.D.			26.43	11.92	0.33		12.94	7.896	15.03	7.90	0.27	11.28
S.E.			9.345	4.179	0.115		4.890	2.984	5.680	2.985	0.119	3.98
4. Obese group												
26. A.M.	34	M	152.7 *111.3	179.0	2.62	60.1	83.71	54.82	68.99	45.18	2.21	18.0
27. R.A.	12	M	67.4 * 25.7	149.8	1.63	38.3	53.34	79.14	14.06	20.86	0.98	25.9
28. T.A.	17	M	143.6 *117.9	170.0	2.46						1.56	22.1
29. C.S.	14	F	96.3 * 63.1	163.8	2.02	32.2	44.85	46.57	51.45	53.43	1.58	28.8
30. H.L.	31	F	58.2 * 4.1	160.0	1.60	32.4	45.12	77.53	13.08	22.47	1.50	18.7
31. A.I.	24	F	97.0 * 73.5	160.0	1.99	36.7	52.36	53.98	44.64	46.02	1.84	21.4
32. C.R.	20	F	95.9 * 87.6	151.1	1.90	34.2	47.63	49.67	48.27	50.33	1.10	13.2
33. M.S.	14	F	92.9 * 60.9	162.5	1.97	36.0	50.17	54.00	42.73	46.00	1.52	30.5
range	12-34		58.2-152.7	149.8-179.0	1.60-2.62	32.2-60.1	44.85-83.71	46.57-79.14	13.08-68.99	20.86-53.43	0.98-2.21	13.2-30.5
mean	20.8		100.5	162.0	2.02		53.88	59.39	40.46	40.61	1.54	22.3
S.D.			32.90	9.50	0.36		13.56	13.27	20.34	13.27	0.39	5.8
S.E.			11.632	3.359	0.127		5.125	5.015	8.295	5.015	0.137	2.06

* These figures represent percent increase above ideal weight in the obese group. The range is 4.1-117.9% and the mean is 68.01%.

Table (26)

Coefficient of correlation between CPR and weight, height, surface area, creatinine excretion, total body lean cell mass and total body fat.

x	y		r_{xy}
1. CPR: blood method (mg./24 hrs.)	Weight	(Kg.)	- 0.189
2. " " "	Height	(cm.)	+ 0.134
3. " " "	Surface area	(m^2)	- 0.119
4. " " "	Creatinine excretion	(g./24hrs)	- 0.207
5. " " "	Total body lean cell mass	(Kg.)	- 0.255
6. " " "	Total body lean cell mass (% of total body weight)		- 0.065
7. " " "	Total body fat	(Kg.)	- 0.145
8. " " "	Total body fat	(up to 30 Kg.)	+ 0.791
9. " " "	Total body fat (% of total body weight)		+ 0.065
10. " " "	Total body fat (% of total body weight; up to 35%)		+ 0.823

A wide scatter and a poor correlation exists between cortisol production rate and weight. The scatter is, however, less pronounced in the normal group alone. Similarly, scatter and poor correlation exists when cortisol production rate is plotted against either the height, surface area, total body lean cell mass (expressed in Kg. or as a percent of the total body weight), total body fat (expressed in Kg. or as percent of total body weight) or creatinine/

creatinine excretion. Again the scatter among the normal group alone is less pronounced, when the surface area or the total body fat are examined, but an interesting observation was the finding of a nearly linear relationship between cortisol production rate and total body fat, if only the subjects of the normal and obese groups whose body fat was 30 Kg. or less were included (see figure 32); the coefficient of correlation for this group was + 0.791, and the straight line in figure 32 corresponds to the regression equation:

$$\underset{\text{(blood method, mg./24hrs)}}{\text{Cortisol production rate}} = 0.576 \times \underset{\text{in Kg.}}{\text{(total body fat)}} + 16.022$$

Similarly, a nearly linear relationship exists between cortisol production rate and the total body fat if the latter is expressed as percent of the total body weight, if only subjects in the normal and obese groups whose body fat is 35% or less of the total body weight are included. The coefficient of correlation in this case is + 0.823, and the straight line in figure 33 corresponds to the regression equation:

$$\underset{\text{(blood method, mg./24hrs)}}{\text{Cortisol production rate}} = 0.577 \times \underset{\substack{\text{as \% of total} \\ \text{body weight}}}{\text{(total body fat)}} + 11.076$$

<u>Conclusion</u>

The conclusions to be drawn from this study are; (1) that poor correlation exists between the adrenocortical function and body weight, height, surface area, lean cell mass or body musculature (i.e. creatinine excretion); and (2) that there is a correlation between the adrenocortical function and total body fat, if the latter is only up to 30 Kg. or 35% of the total body weight.

This, could mean that fat constitutes a mechanical stress to which the adrenals/

adrenals respond by increase of cortisol production. The greater the total body fat, the more cortisol is produced, up to a limit, beyond which the adrenals fail to cope with this extra mechanical stress. Szenas and Pattee (1959) stated that a subject is considered overweight when there is 30% or more increase in actual body weight, in relation to ideal weight.

According to the findings of the present research, obesity as a clinical entity could be defined as "the clinical condition in which the total body fat is 30 Kg. or more" or "the clinical condition in which the total body fat is 35% of the total body weight or more".

Part IV

General Discussion

A. Comparison of the methods of assessment of adrenocortical function used in this study

Taking the group of patients with Cushing's syndrome and the group of normal subjects as the two "reference groups", the most sensitive test of detecting normal or increased adrenocortical activity under basal conditions was estimation of cortisol production rate. Results obtained with the blood method were found to be better than those obtained in the urine method in achieving a separation of these two groups.

The possible reasons for the occasional marked difference between the results of the blood method and urine method include the presence of a circadian rhythm of adrenocortical activity, incomplete collection of urine samples, and the differences in calculation of results between single and double compartment models. Under carefully controlled conditions, urine collection should be complete, and it has been shown that the differences between single and double compartment models play only a minor role at basal conditions. It would seem, therefore, that the main cause of this discrepancy must be the diurnal variation of adrenocortical activity. When Peterson and Wyngaarden (1956), who first introduced the isotope method of CPR estimations, repeated their determinations in the evening, they obtained values which were approximately half of those which they obtained in the morning for the same patients, and this finding has since been confirmed by other workers (Hoet et al, 1961).

In the present thesis, CPR estimated by the urine method was higher than in the blood method in 2 out of 4 cases of Cushing's syndrome (see table 12 a), and this serves to confirm previous reports that the normal diurnal rhythm is absent in patients suffering from this syndrome.

Comparing/

Comparing cortisol production rate on the one hand to steroid excretion on the other have shown that the former is much more sensitive in detecting abnormal adrenocortical function than the latter; this applies particularly to urinary 17-KS estimations. This is not unexpected since it is well known that 17-KS and total 17-KGS methods can be affected by the percentage of cortisol recovered in urine as 17-KS or total 17-KGS, the presence or absence of renal insufficiency or, as recently suggested, by the rate of secretion on the day before (Lazarus, 1962). The last mentioned factor will be important particularly in unsteady conditions of adrenocortical function such as that seen in Cushing's syndrome.

Although estimations of total cortisol level in plasma have provided encouraging results in the present research, as to the diagnosis of normal and hyperactive states of the adrenal cortex, much larger number of cases of Cushing's syndrome need to be assessed by this method before finally accepting this conclusion. The main drawbacks of this method for assessing adrenocortical function should be taken into account, also. These are: (1) total plasma level of cortisol is a measure of the combined effects of cortisol production rate, rate of cortisol disposal and the volume of distribution; (2) plasma cortisol level can be affected by temporary emotional upset; and (3) only the non-protein-bound moiety is regarded nowadays by most workers as the physiologically active component at the tissue or cellular level.

The present findings agree with those reported by Prunty (1961). He studied cases of Cushing's syndrome due to adrenal hyperplasia by various tests and reported that 17-KS exceeded the upper limit of normal in only 6 of 12 patients, 17-KGS raised in 2 of 6 patients, urinary excretion of unmetabolised/

unmetabolised cortisol raised in 4 out of 6 cases, and plasma cortisol levels raised in 2 of 3 cases, but that cortisol production rate estimations were raised in all the 6 patients studied by this method. He concluded that determinations of CPR provided the best available diagnostic criterion.

Recently, in a similar study, Brooks et al (1963) found that 17-KS excretion was increased in only 9 out of 25 cases of Cushing's syndrome, 17-KGS excretion increased in 13 out of 25 cases, plasma cortisol levels increased in 9 out of 17 cases, urinary unmetabolised cortisol excretion increased in 17 of 21 cases but cortisol secretion rate increased in all cases studied by this method.

Estimation of plasma NPB cortisol, in the present study, succeeded in separating clearly a case of Cushing's syndrome from normal and obese subjects. The importance of this parameter as an extremely sensitive index for the assessment of adrenocortical function has been recently stressed by Doe et al (1963). More cases of Cushing's syndrome, however, are needed to decide whether or not the rise of NPB cortisol is a characteristic finding of all patients suffering from this clinical condition.

It has been shown that the discrepancy between CPR estimations on the one hand, and urinary steroid excretion on the other, is even more marked after ACTH, and that there are cases with very high CPR but only moderately increased total 17-KGS excretion. It has also been shown, from studying steroid excretion patterns, that the number of doses of ACTH on the third day of stimulation is not the responsible factor. Lazarus (1962) suggested/

suggested that, during ACTH stimulation, secretion of cortisol on the day before that of estimation of urinary steroids could influence the results considerably. Very recently, James and Caie (1964) found that the correlation between urinary total 17-KGS excretion (as determined by the method of Few, 1961) and secretion rate of cortisol was acceptable in the normal range, but became poor as the secretion rate increased above 35 mg./24 hrs. These workers proved that this discrepancy was not due to increased faecal excretion of cortisol and its metabolites or increased conversion of cortisol to 17-KS after ACTH stimulation. They also proved that the accuracy of the determination of total 17-KGS was not less at high excretion levels. They suggested, therefore, that it is likely that 6β-hydroxycortisol, 16-hydroxylated or other polar metabolites of cortisol may be formed, or their excreted amount increased, at high levels of cortisol secretion, and that probably they may not be extracted from the urine during the assay procedure. These workers emphasised, therefore, the fact that urinary steroid excretion within the normal range can not necessarily be taken as evidence for the exclusion of adrenocortical hyperfunction.

In the present research, the best single test that provided the widest separation between normal subjects and patients with Cushing's syndrome was the CPR after ACTH stimulation. The blood method provided a much wider separation than the urine method. The latter method, it was noticed, always had values well below the former. As previously mentioned, the number of doses of ACTH on the third day of stimulation can not be responsible for this discrepancy, and the answer may lie in finding out whether or not estimations by/

by the blood method from a double compartment model will yield results comparable to that of the urine method.

In the hands of other workers, the results of ACTH stimulation have been very variable. In some occasions, hypersensitivity has been noticed in Cushing's syndrome, and in other instances it has been absent. Many factors are involved in the ACTH test which will decide whether the results obtained will or will not be of any significance. Bayliss and Steinbeck (1954) pointed out the importance of the route of administration of ACTH, and the timing of the plasma samples withdrawn in relation to the beginning of ACTH administration. They noticed that intravenous infusion of 20 units leads to an almost immediate rise of plasma cortisol and a plateau which lasts to the end of the infusion, i.e. for eight hours. The same amount of ACTH given intramuscularly in the form of gel, in one dose, leads to a gradual rise of plasma cortisol and a peak after about 4 hours, whereas injection of 20 units of ACTH intramuscularly, in saline, leads to a more rapid rise of plasma cortisol with a peak level after about 2 hours. These workers also showed that repeated daily injections of ACTH gel led to progressive increase in plasma cortisol, with a maximum level reached on the third day. Forsham et al (1955) stressed the fact that the type of ACTH administered is very important; impure preparations were inactivated when given intramuscularly, but this does not occur with newer preparations. The amount of ACTH injected is also very important.

It has been shown in the present work that the type of test to be applied, after ACTH stimulation, for the assessment of adrenocortical function is also of great importance; CPR estimations were much more superior to urinary steroid excretion estimations.

Beck/

Beck et al (1962) stated that 20 I.U. given intramuscularly every 6 hours for 8 doses, i.e. a total of 160 I.U. over a period of two days, is necessary to give maximal adrenocortical stimulation. These workers summarised the factors which affect the results of the ACTH test, as follows:-

1. The type of ACTH administered.
2. The amount of ACTH administered.
3. The route of administration of ACTH.
4. The duration of administration of ACTH.
5. The test for assessment of adrenocortical function applied after ACTH stimulation.
6. Timing of the samples both as to time of day and their relation to the beginning of ACTH administration.

They demonstrated the importance of these factors in the differentiation of normal subjects from patients with Cushing's syndrome. Using the above mentioned dose, Beck et al (1962) found that at the end of the 48 hour period of the test, a significant difference existed between the means of these two groups as shown by urinary 17-hydroxycorticosteroid estimations. Estimating the plasma 17-hydroxycorticosteroid, the same result was obtained, namely that patients with Cushing's syndrome gave significantly higher results than normals. The means were 35.5 ± 7.7 µg./100 ml., 37.8 ± 12.3 µg./100 ml. and 62.1 µg./100 ml. for normal males, normal females and patients with Cushing's syndrome respectively. The increase above the control value was also significant, 21.2 µg., 24.0 µg. and 41.0 µg. respectively. But when the values obtained four hours after administration of the first injection of ACTH were considered, the results were 38.5 ± 2.5 µg./100 ml., 31.2 ± 5.2 µg./100 ml. and 40.3 ± 4.7 µg./100 ml. respectively; the difference between these means was not significant.

Similarly,/

Similarly, Jailer (1962) noticed a hyper-response to ACTH at the end of the first day of stimulation in 45 out of 46 of his patients with Cushing's syndrome and hyperplasia. The one patient who showed normal response demonstrated the characteristic hyper-response on the third day of stimulation.

In the present research, a unique dose (60 I.U.) of a pure preparation of ACTH gel was given intramuscularly every 12 hours for 3 days, i.e. a total of 360 I.U. over 3 days. This dose is greater that that which has been reported by others to give maximal adrenocortical stimulation. The intramuscular route was chosen because it was the safest. This dose produced maximal stimulation of the adrenal cortex as shown by study of the steroid excretion patterns. Blood samples necessary for estimations of CPR and plasma cortisol levels were withdrawn on the morning of the third day, and the urine samples collected on the third day served for estimation of CPR by the urine method, and for steroid excretion estimations. By using this dosage of ACTH, separation of normal subjects from Cushing's syndrome was achieved with the CPR estimations (much wider in the blood method), but urinary total 17-KGS and 17-KS excretion were not of great help in this respect.

Perhaps the various ways of administering ACTH have been responsible for the contradictory results of this test reported by various workers. The author, therefore, suggests the use of ACTH in the manner applied in the present research, to achieve the best results as far as the diagnosis of cases of Cushing's syndrome is concerned.

B./

B. The importance of grouping the patients in the differentiation of obesity from Cushing's syndrome

The main purpose of the present research has been to achieve a laboratory means of diagnosing Cushing's syndrome, and of separating patients suffering from this condition from normal and obese subjects. This aim has been achieved, first by the choice of sensitive tests of adrenocortical function, but, perhaps of more importance, by classification of the subjects of this study into four groups: (1) normal subjects; (2) patients diagnosed clinically, by laboratory tests, by examination of their adrenals removed at operation and/or the favourable response after adrenalectomy as suffering from Cushing's syndrome (these two groups represented the two "reference" groups; the first represented normal adrenocortical activity and the second represented hyperfunction of the adrenal cortex); (3) "Simple obese" subjects were classified into a separate group, and (4) all the cases which, besides obesity, showed some of the clinical features of Cushing's syndrome were classified into another group which was called 'Cushingoid' group.

The existence of these 'Cushingoid' subjects was recognised many years ago. Thus, Gray et al (1956) described certain "obese individuals with symptoms suggesting Cushing's syndrome". Unfortunately, however, no attempt has been made before to classify the subjects into these four groups. It could well be, therefore, that many of the earlier estimations carried out in "Cushing's syndrome" included obese subjects, which could explain the occasional report of normal adrenocortical activity in Cushing's syndrome, as measured even by sensitive tests such as CPR. It could also explain the inclusion of patients with Cushing's syndrome among investigations of obese subjects,/

subjects, and hence also the frequent reporting of increased adrenocortical activity in obese individuals. The following examples will help to illustrate the importance of this classification if distinct separation of Cushing's syndrome and obesity is aimed at.

Liddle (1960), after classifying his subjects into normal, Cushing's syndrome due to hyperplasia and Cushing's syndrome due to tumours, recognised the presence of another two groups. In one of these two groups, (65 patients) the patients had features "mimicking those of hyperadrenocorticism but with normal basal steroid excretion levels"; these were not considered by Liddle to have Cushing's syndrome because they responded markedly to dexamethasone. In this group central obesity was present in 12, ecchymosis in 3, striae in 7, hypertension in 30, osteoporosis in 9, oedema in 10, emotional instability in 24, oligomenorrhoea in 21, muscle weakness in 6 and hirsutism in 44. The other group included patients with equivocal evidence of Cushing's syndrome. The clinical evidence alone was not strongly suggestive of the syndrome. Control steroid excretions were above normal but this group was again excluded because marked suppression by dexamethasone occurred. Again, in this group of 14 patients, central obesity was present in 3, glucose tolerance impaired in 4, hypertension in 5, osteoporosis in 1, striae in 3, emotional instability in 3, oligomenorrhoea in 2, and hirsutism in 2. Liddle (1960), however, noticed that steroid excretions later on fell to normal in 4 of these cases, and that in none had there been any progression towards unequivocal Cushing's syndrome.

Simkin and Arce (1962) reported their findings of increased 17-KGS excretion in an "obese group", but stated that striae were present in all of them/

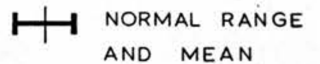
them, acne in 28%, seborrhoea in 24%, plethora in 21%, hirsutism in 34%, oligomenorrhoea in 41%, trunk obesity with dorsal hump in 24%, systolic hypertension in 31%, diastolic hypertension in 21% and hyperglycaemia in 12%.

Baird (1963), similarly, reported increased urinary 17-KGS and 17-KS excretion in an "obese group" of 20 females. Trunk obesity was present in 4, hypertension in 10, impaired carbohydrate tolerance in 2, and oligomenorrhoea or amenorrhoea in 5. Baird stated that Cushing's syndrome was suspected in 11 of these 20 patients. 5 of these 11 obese subjects who had also a clinical abnormality suggestive of an endocrine basis for their obesity, had a raised corticosteroid output. In this group of 5 patients, including the patients with pink striae, the diagnosis of Cushing's syndrome was "strongly doubted" but the results of steroid excretion were nevertheless reported for all these patients, as one group.

Recently, Schteingart et al (1963), comparing 32 "obese subjects" and 23 patients with Cushing's syndrome, reported significantly increased urinary 17-hydroxycorticosteroid excretion and significantly increased CPR in the obese subjects as compared with normal individuals. These workers stated that 3 of their patients, initially considered to have obesity without Cushing's syndrome, later demonstrated deterioration of their clinical condition and a diagnosis of Cushing's syndrome was eventually established in all of these three.

These are a few of the many examples that could be cited from the literature where no effort has been made to classify the subjects strictly into patients with Cushing's syndrome and obese groups, when seeking to interpret experimental results.

By/



NORMAL RANGE AND MEAN
o CUSHING'S
× CUSHINGOID
△ OBESE

CPR BLOOD METHOD — 0, 20, 40, 60 mg/24 hr (→177)

CPR URINE METHOD — 0, 20, 40, 60 mg/24hr (→107)

URINARY 17-KS — 0, 10, 20, 30 mg/24 hr

URINARY TOTAL 17-KGS — 0, 20, 40, 60 mg/24hr

TOTAL PLASMA CORTISOL — 0, 10, 20, 30 µg/100 ml

PLASMA NPB CORTISOL — 0, 2, 4, 6 µg/100ml

CONTROL ESTIMATIONS

CPR BLOOD METHOD — 0, 200, 400, 600 mg/24hr

CPR URINE METHOD — 0, 200, 400, 600 mg/24hr

URINARY 17-KS — 0, 10, 20, 30, 40 mg/24hr

URINARY TOTAL 17-KGS — 0, 100, 200 mg/24hr

TOTAL PLASMA CORTISOL — 0, 20, 40, 60, 80, 100, 120 µg/100 ml

PLASMA NPB CORTISOL — 0, 10, 20, 30 µg/100ml (×→74)

ESTIMATIONS AFTER ACTH

FIGURE 35: COMPARISON OF RESULTS IN THE FOUR GROUPS STUDIED

By classifying the subjects of this study into four groups it was possible to achieve the following results (see tables 27-30 and figure 35):

1. Adrenocortical hyperactivity under basal conditions was proved in all cases of Cushing's syndrome, especially so by the CPR estimations. Adrenocortical hypersensitiveness to ACTH administration was similarly proved. The response to dexamethasone was marked in all cases except one. The cases of Cushing's syndrome studied in this research can be separated into 2 groups.

The first group is represented by one patient (J.A., case 10). In this patient florid clinical manifestations of Cushing's syndrome, together with a markedly increased basal activity of the adrenal cortex (as measured by CPR, urinary steroid excretion, plasma cortisol and plasma NPB cortisol) were noticed. After ACTH, although the level of his CPR was far above the normal group, it represented only about two-fold increase from his control estimations. Similarly, a slight response to dexamethasone suppression was noticed. This patient represented the "classical" picture often described in text books to illustrate the clinical condition and the state of the adrenal cortex in cases of Cushing's syndrome.

The other group, comprising the rest of the patients with Cushing's syndrome, had slightly or moderately increased basal adrenocortical activity, but marked sensitivity to both ACTH stimulation and dexamethasone suppression. Their levels of CPR after ACTH stimulation were the highest observed and were at least 10 times the control values, and the levels after dexamethasone suppression were very low, being at most one tenth of the control values.

The results of the examination of the adrenal glands removed after operation/

operation and the response of the patients to surgery illustrates the fact that the text-book examples of cases of Cushing's syndrome are not as frequent as they are thought to be.

In the group of patients with Cushing's syndrome as a whole, it was possible, by studying the pattern of excretion of urinary steroids, to demonstrate marked day-to-day fluctuation of adrenocortical activity in comparison to normal subjects (see tables 17 and 18 and figures 22 and 23).

2. Cases of simple obesity showed CPR within the normal range in the control estimations, but a lower-than-normal range was obtained after ACTH stimulation. The response of obese subjects to ACTH stimulation, as measured by urinary steroid excretion, was significantly less than in normal individuals. The hyposensitivity of obese subjects to ACTH, in contrast to the hypersensitivity of patients with Cushing's syndrome, adds to the importance of the ACTH test in the differential diagnosis of these two conditions.

The suggestion that adrenocortical function is not hyperactive in obesity was recently supported by Gogate and Prunty (1963), who studied obese patients by various tests of adrenocortical function (17-KS, 17-KGS, unmetabolised cortisol excretion, plasma cortisol level, cortisol secretion rate, ACTH stimulation and dexamethasone suppression) and found that in no subject were two indices of steroid metabolism simultaneously abnormal.

In contrast to cases of Cushing's syndrome, day-to-day fluctuations of adrenocortical activity in the obese group were found to be slight, i.e. much the same as in normal subjects (see tables 17 and 18 and figures 22 and 23).

3. In the 'Cushingoid' group, the results of these investigations allowed separation/

separation into three categories:

(a) This category is represented by patient A.D. (case 18), who showed slightly increased basal adrenocortical activity, as measured by CPR, but marked response to ACTH stimulation, to well within the range for Cushing's syndrome. On repetition of these tests 5 months later, when the clinical condition deteriorated (see table 16), the control estimations did not change significantly but the hyper-response to ACTH was more marked. Therefore, it was justifiable to re-classify this patient as having Cushing's syndrome, and to deduce that the ACTH test was more sensitive in revealing Cushing's syndrome than the clinical condition and that the CPR test by the blood method is superior to any other test in achieving this result.

(b) The second category includes 5 patients (T.M., case 21; C.A., case 22; D.S., case 23; M.K., case 24 and D.Y., case 25). In these patients, values for all tests applied comparable to normal values were obtained, especially after ACTH stimulation. Accordingly, again, it was considered justifiable to re-classify them into the simple obese group. These patients, like the other obese individuals, were put on a reducing diet.

(c) The true 'Cushingoid' group, or group of patients in whom a diagnosis of Cushing's syndrome or of simple obesity could not be made with any certainty was, therefore, reduced to only two patients (W.C., case 19, and D.M., case 20). These represent the third category of the "doubtful" cases. In the former patient, normal control estimations were associated with a hyper-response to ACTH by the CPR (blood method). The caloric intake of this patient, however, was in the region of 12,000 calories per day. In the latter patient moderately elevated control CPR was associated with a normal response/

response to ACTH. The values for his plasma cortisol and NPB cortisol, under basal conditions, were elevated, but only the plasma cortisol level was increased above normal after ACTH stimulation.

Classifying subjects into these four groups, therefore, made it possible to separate Cushing's syndrome from obese subjects, and to reduce the number of the 'Cushingoid' group from eight to two.

The interpretation of the findings in the two patients who remained in the 'Cushingoid' group is a matter for speculation. Perhaps some obese but otherwise normal individuals have abnormally high adrenocortical activity, and perhaps others are unduly responsive to stress and to ACTH stimulation. On the other hand, while the fully developed clinical appearance of simple obesity and of Cushing's syndrome are well known, nothing is known of the adrenal activity during the months or years leading up to the complete syndrome, and it is possible that an increased adrenocortical basal activity, or hypersensitive response to ACTH, is an early manifestation of Cushing's syndrome. These two patients were put on a weight-reducing diet and it is planned to repeat the tests, if considerable weight-reduction, or alternatively more progress of the clinical conditions towards Cushing's syndrome occurred. It is, therefore, anticipated that, in these two cases, typical findings of one or the other clinical conditions may develop in due time.

As a result of this classification, therefore, it is concluded that precise tests of adrenocortical function, such as CPR estimations especially after ACTH, are quite reliable in the differential diagnosis of obesity and Cushing's syndrome; normal results should exclude the latter and higher than normal results in an obese subject should lead to suspicion of the presence of Cushing's syndrome and warrant a careful follow-up for the development of further/

further clinical features of that syndrome.

As already mentioned, other workers have found the clinical features alone very misleading, since many of the signs or symptoms are common to obesity and Cushing's syndrome. Similarly, in the present study, inspite of the association of a greater number of signs and symptoms with Cushing's syndrome than with obesity, no specific clinical manifestation was consistently present in one particular group (see Appendix 1). For example, trunk obesity, although present in all 8 cases of Cushing's syndrome, was encountered in 4 of the 8 obese subjects. Plethora was present in 3 patients with Cushing's syndrome and 2 obese patients. Striae were found in all cases with Cushing's syndrome (purple, pink or red in 6 and white or pale in 2), but were also found in 6 of the obese group (purple, pink or red in 5 and white in 1). Oedema was present in a patient with Cushing's syndrome and in an obese subject, hypertension in 4 cases of Cushing's syndrome and one obese patient, acne or skin infection in 3 patients with Cushing's syndrome and one obese subject and osteoporosis in one patient with Cushing's syndrome and one obese subject. Menstrual disturbances were reported by 4 of the 5 females with Cushing's syndrome (amenorrhoea and menorrhagia) and by 3 obese females (amenorrhoea and irregular periods). Psychological disturbances were also met with in 4 cases of Cushing's syndrome (depression and instability) and in an obese subject (depression and irritability). Diabetes mellitus, however, was not discovered in any of the obese group and was found in only one patient with Cushing's syndrome (elevated fasting blood sugar, delayed glucose tolerance and glycosuria). Similarly, easy bruisability/

bruisability was not found in any of the obese subjects but was found in one case of Cushing's syndrome, and muscle weakness was observed in only two other cases. Varying degrees of hirsutism were met with in 4 of the 5 females with Cushing's syndrome (in 3 of whom male pubic distribution of hair was observed), and this was not seen in any of the obese subjects, but hirsutism was observed in 3 of the 'Cushingoid' group who were subsequently re-classified into the obese group (in one of them a male pubic distribution of hair was observed).

The haematological findings and electrolyte levels in blood were within normal limits in all the subjects studied.

Therefore, it can be concluded that the present findings support previous reports on the unreliability of the clinical features and the non-steroid laboratory investigations in the differential diagnosis of Cushing's syndrome from obesity, and that precise tests of adrenocortical function are not only reliable but also very essential for the pre-operative differentiation of these two clinical conditions.

Table (27)*

Comparison of results in the four groups studied

Group	CPR (mg/24hrs)		17-KS	Total 17-KGS	Total plasma cortisol	Plasma NPB cortisol (in vitro method)	
	Blood method	Urine method	(mg./24hrs)	(mg./24hrs)	(µg./100ml.)	(%)	(µg./100ml)
a. Control estimations							
1. Normal group	7 17.5-31.2 (25.2±5.3)	7 11.6-29.0 (20.0±7.15)	8 4.0-13.0 (8.9±3.2)	8 7.3-22.0 (14.5±5.65)	6 9.7-15.9 (11.9±3.41)	6 11.1-16.0 (14.1±1.79)	6 1.00-2.39 (1.67±0.52)
2. Cushing's group	4 34.1-177.6 (76.1±68.3)	7 24.6-107.0 (47.7±29.14)	8 10.2-32.7 (16.8±7.69)	8 12.7-54.9 (28.6±15.53)	1 23.3	1 17.9	1 4.2
3. 'Cushingoid' group	8 8.6-45.6 (27.6±11.28)	8 13.4-41.2 (24.0±9.41)	8 2.3-30.0 (11.5±8.68)	8 13.0-30.6 (21.3±7.48)	5 9.3-29.6 (15.8±8.09)	5 10.2-23.0 (16.8±4.62)	5 1.46-4.70 (2.58±1.44)
4. Obese group	8 13.2-30.5 (22.3±5.8)	8 15.5-27.7 (20.8±4.05)	7 5.0-9.2 (7.5±1.58)	7 9.5-22.0 (14.5±4.08)	2 6.4-16.8 (11.6±7.36)	2 12.5-24.4 (18.5±8.41)	2 1.55-2.10 (1.83±0.39)
b. After ACTH							
1. Normal group	5 216-268 (235±22)	6 85-262 (184±67)	5 13.0-31.0 (21.2±0.8)	5 84-125 (115±17)	5 27.6-68.2 (52.6±16.73)	5 25.7-45.5 (36.7±7.63)	5 7.1-27.3 (19.8±8.01)
2. Cushing's group	3 350-631 (494±140)	3 263-450 (356±93)	4 9.7-33.4 (23.3±10.3)	4 56-176 (103±53)	-	-	-
3. 'Cushingoid' group	6 187-450 (277±104)	7 106-225 (149±37)	7 5.0-40.0 (19.1±11.9)	7 50-118 (78±24)	4 38.8-128.1 (75.8±11.93)	4 19.4-58.1 (38.0±15.81)	4 7.5-74.4 (33.2±28.77)
4. Obese group	8 177-268 (193±45)	8 42-242 (108±63)	6 6.6-13.0 (10.4±2.8)	6 33-84 (52±17)	2 53.6-68.5 (61.1±9.0)	2 38.3-40.0 (39.2±1.2)	2 20.5-27.4 (23.9±4.89)

Table (27)* contd.

Group	CPR (mg./24hrs)		17-KS	Total 17-KGS	Total plasma cortisol	Plasma NPB cortisol (in vitro method)	
	Blood method	Urine method	(mg./24hrs)	(mg./24hrs)	(µg./100ml)	(%)	(µg./100ml)
c. After dexam.							
2. Cushing's group	1 33.6	3 3.1-39.5 (15.4±20.8)	2 3.0-4.0 (3.5±0.7)	2 5.2-15.7 (10.4±7.4)	1 9.0	1 7.5	1 0.67
3. 'Cushingoid' group	-	5 1.1-3.7 (2.3±1.0)	4 1.0-6.0 (3.4±2.3)	4 1.0-6.7 (3.8±2.5)	-	-	-
4. Obese group	-	8 1.2-7.8 (3.4±2.0)	7 2.5-7.2 (3.7±1.6)	7 1.1-5.0 (3.7±1.6)	-	-	-

*The ranges are given. The underlined figures represent the number of cases estimated in each group and the figures in brackets represent the mean and S.D.

Table (28)

Statistical analyses of the differences between the means of the various tests among the four groups studied

	Normal Cushing's	Normal Cushingoid	Normal Obese	Cushing's Cushingoid	Cushing's Obese	Cushingoid Obese
a. Control						
Miscible pool (mg.)	1.8 3.9 P= .01**	1.8 2.3 .2 < P < .5	1.8 1.7 P > .5	3.9 2.3 .05 < P < .1	3.9 1.7 P < .01**	2.3 1.7 .05 < P < .1
Rate of turnover (pool/hr)	0.58 0.75 .1 < P < .2	0.58 0.49 .1 < P < .2	0.58 0.62 P > .5	0.75 0.49 .02 < P < .05	0.75 0.62 .2 < P < .5	0.49 0.62 .2 < P < .5
CPR: blood method (mg./24hr)	25.2 76.1 .02 < P < .05*	25.2 27.6 P > .5	25.2 22.3 .2 < P < .5	76.1 27.6 .05 < P < .1	76.1 22.3 .02 < P < .05*	27.6 22.3 .2 < P < .5
CPR: urine method (mg./24hr)	20.0 47.7 .02 < P < .05*	20.0 24.7 .2 < P < .5	20.0 19.4 P > .5	47.7 24.2 .05 < P < .1	47.7 19.4 .01 < P < .02*	24.7 19.4 .1 < P < .2
Dose excretion; first 24 hrs. (%)	78.9 81.6 P > .5	78.9 79.6 P > .5	78.9 79.5 P > .5	81.6 79.6 P > .5	81.6 79.5 P > .5	79.6 79.5 P > .5
17-KS (mg./24hr)	8.9 16.8 .01 < P < .02*	8.9 11.5 .2 < P < .5	8.9 7.5 .2 < P < .5	16.8 11.5 .05 < P < .1	16.8 7.5 P < .01**	11.5 7.5 .2 < P < .5
Total 17-KGS (mg./24hr)	14.5 28.6 .02 < P < .05*	14.5 21.3 .05 < P < .1	14.5 14.5 P > .5	28.6 21.3 .2 < P < .5	28.6 14.5 .02 < P < .05*	21.3 14.5 .05 < P < .1
Total plasma cortisol (µg./100ml)	11.9 23.3 .02 < P < .05*	11.9 15.8 .2 < P < .5	11.9 11.6 P > .5	23.3 15.8 .2 < P < .5	23.3 11.6 .2 < P < .5	15.8 11.6 P > .5
NPB cortisol; *in vitro* (µg./100ml)	1.7 4.2 P < .01**	1.7 2.6 .1 < P < .2	1.7 1.8 P > .5	4.2 2.6 .2 < P < .5	4.2 1.8 .1 < P < .2	2.6 1.8 .2 < P < .5
NPB cortisol *in vitro* (%)	14.1 17.9 .1 < P < .2	14.1 16.8 .2 < P < .5	14.1 18.5 .2 < P < .5	17.9 16.8 P > .5	17.9 18.5 P > .5	16.8 18.5 P > .5
NPB cortisol *in vivo* (µg./100ml)		1.37 1.27 P > .5				
NPB cortisol *in vivo* (%)		8.9 11.1 P > .5				

Table (28) contd

	Normal / Cushing's	Normal / Cushingoid	Normal / Obese	Cushing's / Cushingoid	Cushing's / Obese	Cushingoid / Obese
b. After ACTH						
Miscible pool (mg.)	9.9 18.8 $.01<P<.02$*	9.9 15.9 $.05<P<.1$	9.9 8.9 $P>.5$	18.8 15.9 $P>.5$	18.8 8.9 $P<.01$**	15.9 8.9 $.02<P<.05$*
Rate of turnover (pool/hr)	1.00 1.10 $.2<P<.5$	1.00 0.75 $.02<P<.05$*	1.00 1.00 $P>.5$	1.10 0.75 $.01<P<.02$*	1.10 1.00 $P>.5$	0.75 1.00 $.05<P<.1$
CPR: blood method (mg./24hr)	235 494 $P<.01$**	235 277 $.2<P<.5$	235 193 $.05<P<.1$	494 277 $.02<P<.05$*	494 193 $P<.01$**	277 193 $.05<P<.1$
CPR: blood method. Increase over control (mg)	210 404 $P<.01$**	210 246 $.2<P<.5$	210 171 $.05<P<.1$	404 246 $.1<P<.2$	404 171 $P<.01$**	246 171 $.05<P<.1$
CPR: blood method. Increase over control (%)	882 791 $P>.5$	882 900 $P>.5$	882 805 $P>.5$	791 900 $P>.5$	791 805 $P>.5$	900 805 $P>.5$
CPR: urine method (mg./24hr)	184 356 $.01<P<.02$*	184 149 $.2<P<.5$	184 108 $.05<P<.1$	356 149 $P<.01$**	356 108 $P<.01$**	149 108 $.2<P<.5$
CPR: urine method. Increase over control (mg)	168 291 $.2<P<.5$	168 122 $P>.5$	168 89 $.2<P<.5$	291 122 $.2<P<.5$	291 89 $P<.01$**	122 89 $.2<P<.5$
CPR: urine method. Increase over control (%)	1122 782 $P>.5$	1122 521 $.02<P<.05$*	1122 443 $.01<P<.02$*	782 521 $.2<P<.5$	782 443 $.2<P<.5$	521 443 $P>.5$
Dose excretion first 24 hrs. (%)	79.7 73.3 $.2<P<.5$	79.7 83.9 $.05<P<.1$	79.7 78.4 $P>.5$	73.3 83.9 $.1<P<.2$	73.3 78.4 $P>.5$	83.9 78.4 $P>.5$
17-KS (mg./24hr)	21.2 23.3 $P>.5$	21.2 19.2 $P>.5$	21.2 10.4 $P<.01$**	23.3 19.2 $P>.5$	23.3 10.4 $.01<P<.02$*	19.2 10.4 $.1<P<.2$
17-KS: Increase over control (mg)	12.8 9.8 $P>.5$	12.8 8.4 $.2<P<.5$	12.8 3.2 $.02<P<.05$*	9.8 8.4 $P>.5$	9.8 3.2 $.1<P<.2$	8.4 3.2 $.02<P<.05$*
17-KS: Increase over control (%)	217 91 $.2<P<.5$	217 101 $.2<P<.5$	217 45 $.1<P<.2$	91 101 $P>.5$	91 45 $.2<P<.5$	101 45 $.1<P<.2$
Total 17-KGS (mg./24hr)	115 103 $P>.5$	115 78 $.01<P<.02$*	115 52 $P<.01$**	103 78 $.2<P<.5$	103 52 $P=.05$*	78 52 $.05<P<.1$

Table (28) contd

	Normal / Cushing's	Normal / Cushingoid	Normal / Obese	Cushing's / Cushingoid	Cushing's / Obese	Cushingoid / Obese
Total 17-KGS: Increase over control (mg)	98 73 $.2<P<.5$	98 56 $P<.01$**	98 37 $P<.01$**	73 56 $.2<P<.5$	73 37 $.1<P<.2$	56 37 $.1<P<.2$
Total 17-KGS: Increase over control (%)	672 316 $.1<P<.2$	672 285 $.02<P<.05$*	672 272 $.02<P<.05$*	316 285 $P>.5$	316 272 $P>.5$	285 272 $P>.5$
Total plasma cortisol (μg./100ml)		52.6 75.8 $.2<P<.5$	52.6 61.1 $.2<P<.5$			75.8 61.1 $P>.5$
Total plasma cortisol: Increase over control (μg)		41.4 58.4 $.05<P<.1$	41.4 49.45 $P>.5$			58.4 49.45 $P>.5$
Total plasma cortisol: Increase over control (%)		380 388 $P>.5$	380 522 $.2<P<.5$			388 522 $P>.5$
NPB cortisol *in vitro* (%)		36.7 38.0 $.2<P<.1$	36.7 39.2 $P>.5$			38.0 39.2 $P>.5$
% NPB cortisol *in vitro* Increase over control (absol.)		22.0 21.5 $P>.5$	22.0 20.7 $P>.5$			21.5 20.7 $P>.5$
% NPB cortisol *in vitro* Increase over control (%)		151 124 $.2<P<.5$	151 138 $P>.5$			124 138 $P>.5$
NPB cortisol *in vitro* (μg./100ml)		19.8 33.2 $.2<P<.5$	19.8 23.9 $P>.5$			33.2 23.9 $P>.5$
NPB cortisol *in vitro* Increase over control (μg)		18.18 30.36 $P>.5$	18.18 22.13 $P>.5$			30.36 22.13 $P>.5$
NPB cortisol *in vitro* Increase over control (%)		1153 1068 $P>.5$	1153 1213 $P>.5$			1068 1213 $P>.5$
NPB cortisol *in vivo* (%)		32.4 13.5 $.2<P<.5$				
NPB cortisol *in vivo* (μg./100ml)		15.3 5.2 $.2<P<.5$				

Table (28) contd

	Normal Cushing's	Normal Cushingoid	Normal Obese	Cushing's Cushingoid	Cushing's Obese	Cushingoid Obese
c. After dexam.						
CPR: urine method (mg./24hr)				15.4 2.4 .1<P<.2	15.4 3.5 .1<P<.2	2.4 3.5 .2<P<.5
17-KS (mg./24hr)				3.5 3.4 P>.5	3.5 3.7 P>.5	3.4 3.7 P>.5
Total 17-KGS (mg./24hr)				10.5 3.8 .1<P<.2	10.5 3.7 .02<P<.05*	3.8 3.7 P>.5
Dose excretion (%)				79.4 86.5 .1<P<.2	79.4 77.9 P>.5	86.5 77.9 .1<P<.2
Weight (Kg)	59.5 91.8 P<.01**	59.5 94.2 P<.01**	59.5 100.5 P<.01**	91.8 94.2 .1<P<.2	91.8 100.5 P>.5	94.2 100.5 P>.5
Height (cm)	167.6 161.2 .2<P<.5	167.6 165.4 P>.5	167.6 162.0 .2<P<.5	161.2 165.4 P>.5	161.2 162.0 P>.5	165.4 162.0 P>.5
Surface area (m^2)	1.66 1.98 .02<P<.05*	1.66 2.00 .2<P<.5	1.66 2.02 P<.01**	1.98 2.00 P>.5	1.98 2.02 P>.5	2.00 2.02 P>.5
Total body lean cell mass (Kg)	42.43 52.00 .02<P<.05*	42.43 58.30 .02<P<.05*	42.43 53.88 .05<P<.1	52.00 58.30 .2<P<.5	52.00 53.88 P>.5	58.30 53.88 P>.5
Total body lean cell mass (% of total body weight	73.18 57.04 .01<P<.02*	73.18 59.71 .01<P<.02*	73.18 59.39 .05<P<.1	57.04 59.71 P>.5	57.04 59.39 P>.5	59.71 59.39 P>.5
Total body fat (Kg)	16.49 39.81 P<.01**	16.49 40.58 P<.01**	16.49 40.46 .02<P<.05*	39.81 40.58 P>.5	39.81 40.46 P>.5	40.58 40.46 P>.5
Total body fat (% of total body weight)	26.82 42.96 .01<P<.02*	26.82 40.29 .01<P<.02*	26.82 40.61 .05<P<.1	42.96 40.29 P>.5	42.96 40.61 P>.5	40.29 40.61 P>.5
Creatinine excretion (g./24hr)	1.21 1.43 .2<P<.5	1.21 1.64 .02<P<.05*	1.21 1.54 .1<P<.2	1.43 1.64 .2<P<.5	1.43 1.54 P>.5	1.64 1.54 P>.5

* Significant
** Highly significant

Table (29)

Statistical analyses of the differences between the means of the various tests under control, stimulation and suppression states

	Normal group	Cushing's group	'Cushingoid' group	Obese group
a. Control ACTH Miscible pool (mg.)	1.8 9.9 $P<.01$**	3.9 18.8 $P<.01$**	2.3 15.9 $P<.01$**	1.7 8.9 $P<.01$**
Rate of turnover (pool/hr)	0.58 1.00 $P<.01$**	0.75 1.10 $P=.05$*	0.49 0.75 $P<.01$**	0.62 1.00 $.01<P<.02$*
CPR: blood method (mg./24hr)	25.2 235 $P<.01$**	76.1 494 $P<.01$**	27.6 277 $P<.01$**	22.3 193 $P<.01$**
CPR: urine method (mg./24hr)	20.0 184 $P<.01$**	47.7 356 $P<.01$**	24.7 149 $P<.01$**	19.4 108 $P<.01$**
Dose excretion first 24 hr. (%)	78.9 79.7 $P>.5$	81.6 73.3 $.2<P<.5$	79.6 83.9 $.2<P<.5$	79.5 78.3 $P>.5$
17-KS (mg./24hr)	8.9 21.2 $P<.01$**	16.8 23.3 $.2<P<.5$	11.5 19.2 $.1<P<.2$	7.5 10.4 $.02<P<.05$*
Total 17-KGS (mg./24hr)	14.5 115 $P<.01$**	28.6 103 $P<.01$**	21.3 78 $P<.01$**	14.5 52 $P<.01$**
Total plasma cortisol (µg./100ml)	11.9 52.6 $P<.01$**		15.8 75.8 $P<.01$**	11.6 61.1 $P<.01$**
NPB cortisol *in vitro* (%)	14.1 36.7 $P<.01$**		16.8 38.0 $.02<P<.05$*	18.5 39.2 $.05<P<.1$
NPB cortisol *in vitro* (µg./100ml)	1.7 19.8 $P<.01$**		2.6 33.2 $.02<P<.05$*	1.8 23.9 $P<.01$**
b. Control Dexamethasone CPR: urine method (mg./24hrs)		47.7 15.4 $.1<P<.2$	24.7 2.4 $P<.01$**	19.4 3.4 $P<.01$**
17-KS (mg./24hr)		16.8 3.5 $.02<P<.05$*	11.5 3.4 $.1<P<.2$	7.5 3.7 $P<.01$**
Total 17-KGS (mg./24hr)		28.6 10.5 $.1<P<.2$	21.3 3.8 $P<.01$**	14.5 3.7 $P<.01$**
Dose excretion (%)		81.6 79.4 $P>.5$	79.6 86.5 $.01<P<.1$	79.5 77.9 $P>.5$

*Significant **Highly significant

Table (30)*

Comparison of the results of the various tests in individual cases of the four groups studied

Case	Control							After ACTH							After dexamethasone		
	CPR		17-KS	Total 17-KGS	Total Plasma Cortisol level	NPB cortisol		CPR		17-KS	Total 17-KGS	Total Plasma Cortisol level	NPB cortisol		CPR	17-KS	Total 17-KGS
	Blood	Urine				%	level	Blood	Urine				%	level	Urine		
1. Normal group																	
1. B.A.	−																
2. R.B.	−	−	±	±	−	−	−										
3. W.J.	−	−	−	±	−	−	−	−	−	±	±	−	−	−			
4. W.H.	−	−	±	±	−	−	−	−	−	±	±	−	−	−			
5. M.L.	−	−	±	±	−	−	−	−	−	±	±	−	−	−			
6. M.H.	−	−	−	−	−	−	−	−	−	±	±	−	−	−			
7. M.R.	−	−	±	±	−	−	−	−	−	±	±	−	−	−			
8. T.T.		±	±	−					−								
9. S.F.		±	±	−													
2. Cushing's group																	
a. Operated upon																	
10. J.A.	+	+	+	+	+	+	+	+	+	±	±				+	±	+
11. J.T.	+	+	+	±				+	+	+	±				±	±	+
12. M.C.	+	±	±	±				+	+	±	+				±		
13. J.M.		+	+	+													
14. G.F.			+	+													
15. H.T.		±	±	±													
16. E.S.		+	±	±													
b. Not operated upon																	
17. C.M.	+	+	±	±						±	±						
3. 'Cushingoid' group																	
a. Possibly Cushing's																	
18. A.D.	+	+	−	+				+	−	−	±				±	−	−
18. A.D.**	−	+	−	±	−	+	±	+	+	±	±	+	+	+	−	−	−
b. Doubtful																	
19. W.C.	−	−	±	±				+	−	±	±				−	−	+
20. D.M.	+	+	+	+	+	±	+	−		+	±	+	−	−	−	−	−
c. Possibly obese																	
21. T.M.	−	±	±	+				−	−	±	±				±		
22. C.A.	−	−	+	±													
23. D.S.	−	−	±	±	−	−	−	−	−	−	±	−	−	−	−	−	−
24. M.K.	−	±	±	±	−	−	−	−	−	±	±	−	−	−			
25. D.Y.	−	−	−	±	−	±	−		−	±	−						
4. Obese group																	
26. A.M.	−	−						−	−						±		
27. R.A.	−	−	±	−				−	−	±	−				−	±	−
28. T.A.	−	−	±	±	−	+	−	−	−	±	±	−	−	−	−	±	−
29. C.S.	−	±	±	±				−	−	−	±				−	−	−
30. H.L.	−	−	±	±				−	−	±	−				−	±	−
31. A.I.	−	−	−	±	±	−	−	−	−	−	−	−	−	−	±	±	−
32. C.R.	−	−	±	−				−	−						±	±	−
33. M.A.	−	−	±	±				−	−	±	−				−	±	−

* − represent figures in the normal range, + represent figures in the Cushing's range, ± represent figures in overlapping ranges. After dexamethasone suppression

Suggestions for future work

The author suggests the following points to be investigated in future work:

1. Investigations into the discrepancy between the blood and the urine method of CPR after ACTH stimulation. This should comprise firstly, estimations of the excretion of 6 β-hydroxycortisol and other polar steroids and secondly, the volume of distribution and whether estimations of CPR from a double compartment model, instead of from a single compartment model, would explain this discrepancy.

2. Investigations into the value of estimations of unmetabolised cortisol excretion as a possible important means of diagnosing Cushing's syndrome.

3. Establishment of ranges for the *in vivo* method of simultaneous estimation of CPR, plasma cortisol and plasma-protein binding of cortisol in a larger number of subjects from the four groups (normal, Cushing's syndrome, 'Cushingoid' and obese). The author recommends the routine determination of plasma proteins in the sample used for estimation of NPB cortisol; allowance for the volume of plasma proteins will, therefore, be made, and accordingly the results of estimations of NPB cortisol will be more accurate.

4. Investigation into the question of whether or not a circadian rhythm in plasma cortisol and the NPB fraction normally exists, and whether or not it is always lost in cases of Cushing's syndrome. This may prove to be of great help in the early diagnosis of Cushing's syndrome.

5. Estimation of CPR and steroid excretion after dexamethasone suppression in a large number of normal subjects, and in cases of Cushing's syndrome, for the/

the assessment of the value of this test in diagnosis.

6. The electron microscopic study of each adrenal gland removed from cases of Cushing's syndrome, together with ordinary histological examination, with the aim of detecting any abnormality that may not always be detected in glands from patients with Cushing's syndrome by the latter methods.

7. Study of the effect of weight reduction on adrenocortical function in obese subjects. Similarly, in cases of Cushing's syndrome, if the clinical condition is not so rapidly progressive as to warrant an immediate surgical interference, it would be worthwhile to determine the effect of weight reduction over a few weeks or a few months (according to the progress of the condition) on adrenocortical function, and to compare this with the response of obese subjects to a similar regimen.

Appendix I

Case reports

(see Appendix II for abbreviations)

The following are the normal ranges for the methods used:-

serum or plasma

Sodium	135 - 150 m.eq/l.
Potassium	3.5 - 5.5 "
Bicarbonate	22.0 - 28.0 "
Blood urea	14.0 - 40.0 mg./100 ml.
Calcium	9.0 - 11.0 "
Phosphate	2.5 - 4.0 "
Fasting blood sugar	60 - 95 "
Bilirubin	0.1 - 0.8 "
Glutamic-pyruvic transaminase	5 - 35 units/ml.
Alkaline phosphatase	3 - 12 King-Armstrong units/100 ml.
Thymol Turbidity	1 - 5 units.
Creatinine clearance	90 - 150 ml/min.

I Normal group

Case No.1 B.A. Sex: M Age: 29 yrs.

Normal volunteer

Wt: 63 Kg. Ht: 175 cm.

Case No.2 R.B. Sex: M Age: 65 yrs.

Diagnosis: Chronic bronchitis

Wt: 63.4 Kg. Ht: 177.8 cm. B.P. $\frac{135}{90}$

Investigations:

Xray chest: mild congestion of lungs

Blood picture: Hb. 114%, PCV 50%, MCHC 32%, WCC 6500, Diff. N 69%, L 26%, M 2%, E 3%.

Electrolytes in blood: Na 136 meq/l, K 4 meq/l, B.U. 27 mg./100 ml.

GPT less than 20 units/ml, Alk.Phos. 10 K-A units/100 ml, T.T. 2 units.

<u>Case No.3</u> W.J. Sex: M Age: 35 yrs.

Diagnosis: Barbiturate overdose, acute renal failure, fully recovered.

Wt: 54 Kg. Ht: 170 cm. B.P. $\frac{125}{85}$

Investigations:

ECG: NAD

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 98%, PCV 48%, MCHC 37%, platelets 350,000, WCC 9000, Diff. N 83%, L 17%.

Electrolytes in blood: Na 140 meq/l, K 4.3 meq/l, HCO_3 20.6 meq/l, B.U. 39 mg./100 ml.

Ca 10.4 mg./100 ml, PO_4 4.9 mg./100 ml.

Bili. 0.8 mg./100 ml. GPT less than 20 units/ml.

Alk.Phos. 11 K-A units/100 ml, T.T. 1 unit.

Cr.cl. 142ml/min.

Urine examination: NAD

<u>Case No.4</u> W.H. Sex: M Age: 55 yrs.

Diagnosis: ? Pericarditis ? myocardial infarction, completely recovered.

Wt: 83.4 Kg. Ht: 171.4 cm. B.P. $\frac{110}{66}$

Investigations:

ECG: ? pericarditis ? myocardial infarction.

X-ray chest: heart enlarged.

Blood picture: Hb. 101%, WCC 10800, Diff. N 80%, L 11%, M 9%.

Electrolytes in blood: Na 140 meq/l, K 5.0 meq/l, HCO_3 23 meq/l

Bili. 0.8 mg./100 ml. GPT 26 units/ml. Alk.Phos. 9 K-A units/100 ml.

Case No.5 M.L. Sex: F Age: 42 yrs.

Diagnosis: Functional polyuria and stress incontinence.

Wt: 44 Kg. Ht: 156.2 cm. B.P. $\frac{120}{80}$

Investigations:

Blood picture: Hb. 96%, PCV 42%, MCHC 34%, platelets 105,100, WCC 6600, Diff. N 69%, L 27%, M 3%, E 1%.

Electrolytes in blood: Na 139 meq/l, K 4.8 meq/l, HCO_3 26.1 meq/l, B.U. 27 mg./100 ml.

Ca 10.0 mg./100 ml.

Alk.Phos. 4 K-A units/100 ml.

Urine examination: NAD

Cr.Cl. 157 ml/min.

Case No.6 M.H. Sex: F Age: 35 yrs.

Diagnosis: Dysuria

Wt: 52 Kg. Ht: 165.1 cm. B.P. $\frac{110}{80}$

Investigations:

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 81%, PCV 38%, MCHC 29%, WCC 8700, Diff. N 75%, L 19%, M 5%, E 1%.

Electrolytes in blood: Na 140 meq/l, K 4.6 meq/l, B.U. 26 mg./100 ml.

Ca 10.6 mg./100 ml, PO_4 3.1 mg./100 ml.

Bili. 0.4 mg./100 ml, GPT less than 20 units/ml, Alk.Phos. 7 K-A units/100ml.

Cr.cl. 67 ml/min.

Case No.7 M.R. Sex: F Age: 23 yrs.

Diagnosis: Acute renal failure, fully recovered.

Wt: 56.7 Kg. Ht: 157.4 cm.

Investigations:

Blood picture: Hb. 68%, PCV 32%, MCHC 31%, WCC 9700, Diff. N 74%, L 20%, M 4%, E 1%, B 1%.

Electrolytes in blood: Na 141 meq/1, K 4.0 meq/1, HCO_3 21.0 meq/1, B.U. 20 mg./100 ml.

Cr.cl. 150 ml/min.

Case No.8 T.T. Sex: M Age: 72 yrs.

Diagnosis: dermatitis herpitiformis.

Investigations:

B.P. $\frac{160}{100}$ X-ray chest: NAD

Blood picture: Hb. 73%, PCV 37%, MCHC 29%, WCC 9000, Diff. N 75%, L 22%, M 1%, E 2%.

Fasting blood sugar 82 mg./100 ml.

Case No.9 S.F. Sex: M Age: 42

Normal volunteer

II Cushing's group

(see also table 24 for results of examination of adrenal glands removed at operation).

Case No. 10 J.A. Sex: M Age: 55 yrs.

a. Symptoms: increased girth of lower limbs and abdomen, swelling of ankles, intermittent claudication and plethoric appearance. Duration 6 months.

b. Examination: Wt: 89 Kg. Ht: 157.4 cm. B.P. $\frac{205}{120}$

Skin: scaly and red. Boils: recurrently occurring.

Obesity: marked, mainly at lower abdominal girth.

Striae: numerous on lower abdomen. Purple. Oedema: at lower limbs.

Face: plethoric. Muscle weakness: marked. Easy bruisability.

c. Investigations:

ECG: NAD.

X-ray chest: considerable cardiac enlargement.

X-ray skeleton: lumbar scoliosis with wedge deformity of L 1-4.

Blood picture: Hb. 95%, PCV 43%, MCHC 32.5%, WCC 10200, Diff. N 75%, L 15%, M 5%.

Electrolytes in blood: Na 141 meq/l, K 4.3 meq/l, HCO_3 25.8 meq/l, BU 51 mg./100 ml.

Ca 12.0 mg./100 ml, PO_4 2.8 mg./100 ml.

Bili. 0.5 mg./100 ml. GPT less than 20 units/ml.

Alk.Phos. 5 K-A units/100 ml, T.T. 1 unit.

G.T.T.: F 73 mg./100 ml, ½hr 95 mg./100 ml, 1 hr 133 mg./100 ml, 1½ hrs. 85 mg./100 ml, 2 hrs. 73 mg./100 ml.

Cr.cl 92 ml/min.

d. Treatment and follow up: Bilateral adrenalectomy and replacement therapy. Marked improvement of clinical picture after operation.

Case No.11 J.T. Sex: F Age: 17 yrs.

a. Symptoms: increase in weight of 32 Kg., striae and menorrhagia in the past 6 months.

b. Examination: Wt: 87 Kg. Ht: 160 cm. B.P. $\frac{150}{110}$

Skin: NAD. Acne: nil. Bruising: nil. Hirsutism: nil.

Obesity: marked, mainly trunk obesity.

Striae: marked. On ~~axeille~~ axillae, breasts, flanks and abdomen. Reddish.

Face: round and full. Oedema: nil. Genitals: normal.

c. Investigations:

ECG NAD

X-ray chest: NAD. X-ray skeleton: NAD.

Blood picture: Hb. 94%, PCV 43%, MCHC 30%, WCC 6700, Diff. N 80%, L 15%, M 4%, L 1%, platelets 270,000.

Electrolytes in blood: Na 133 meq/l, K 4.5 meq/l, HCO_3 24 meq/l, BU 10 mg./100 ml.

Ca 9.4 mg./100 ml, PO_4 3.0 mg./100 ml.

Bili. 0.2 mg./100 ml, GPT 21 units/ml. Alk.Phos. 5 K-A units/100 ml, T.T. 2 units/[illegible]

G.T.T.: F 72 mg./100 ml, ½hr 148 mg./100 ml, 1 hr 144 mg./100 ml, 1½ hrs 120 mg./100ml, 2 hrs 118 mg./100 ml, 2½ hrs 82 mg./100 ml, 3 hrs 60 mg./100 ml.

Cr.cl 162 ml/min.

d. Treatment and follow up: Bilateral adrenalectomy and auto-transplantation. Marked improvement of the clinical condition post-operatively.

Case No.12 M.C. Sex: F Age: 36 yrs.

a. Symptoms: hirsutism, gain in weight of 30 Kg. Duration 10 years.

Amenorrhoea and depression for 6 months.

b. Examination: Wt: 108 Kg. Ht: 166.3 cm. B.P. $\frac{190}{118}$

Skin: NAD. Acne: slight. Bruising: nil. Hirsutism: marked. On cheeks, chin, chest with a pubic masculine distribution.

Obesity: marked. Mainly trunk obesity. Cervico-dorsal hump.

Striae: present. White. On abdomen and thighs. Duration 10 years.

Face: Plethoric, moon face. No muscle weakness.

Genitals: normal. No oedema.

c. Investigations:

ECG: NAD

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 94%, PCV 39%, MCHC 35.5%, WCC 11000, Diff. N 63%, L 35%, M 2%, platelets 380,000.

Electrolytes in blood: Na 140 meq/l, K 4.8 meq/l, HCO_3 18.8 meq/l BU 10 mg./100 ml.

Ca 10.8 mg. /100 ml, PO_4 2.9 mg./100 ml.

Bili. 0.7 mg./100 ml. GPT 31 units/ml.

Alk.Phos. 3 K-A units/100 ml, T.T. 3 units.

G.T.T.: F 72 mg./100 ml, ½ hr 90 mg./100 ml, 1 hr 100 mg./100 ml, 1½ hrs 68 mg./100 ml, 2 hrs 48 mg./100 ml, 2½ hrs 60 mg./100 ml, 3 hrs 60 mg./100 ml.

Cr.cl 169 ml/min.

d. Treatment and follow up: Bilateral adrenalectomy and auto-transplantation.

Marked improvement of clinical condition post-operatively.

Case No. 13 J.M. Sex: M Age: 31 yrs.

a. Symptoms: progressive increase in weight since 5 years. Weakness and Depression since 6 months.

b. Examination: Wt: 116.8 Kg. B.P. $\frac{130}{90}$

Skin: NAD Acne: on chest and back. No bruising.

Thinning of the frontal scalp hair.

Obesity: marked. Mainly trunk. Supraclavicular pads of fat.

Striae: present on the abdomen. Purple.

Face: round and flushed.

No muscle weakness or oedema.

c. Investigations:

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 104%, PCV 46%, MCHC 31.5%, WCC 8800.

Electrolytes in blood: Na 154 meq/l, K 5.3 meq/l,

HCO_3 24.6 meq/l, BU 15 mg./100 ml.

G.T.T.: F 80 mg./100 ml, $\frac{1}{2}$ hr 136 mg./100 ml, 1 hr 100 mg./100 ml,

$1\frac{1}{2}$ hrs 68 mg./100 ml, 2 hrs 72 mg./100 ml, $2\frac{1}{2}$ hrs 68 mg./100 ml.

d. Treatment and follow up: bilateral adrenalectomy and replacement therapy.

Marked improvement of clinical condition post-operatively.

Case No. 14 G.F. Sex: M Age: 27 yrs.

a. Symptoms: progressive increase in weight. Depression.

b. Examination: Wt: 78.8 Kg. Ht. 166.3 cm. B.P. $\frac{140}{105}$

Skin: NAD No acne or bruising.

Obesity: marked. Mainly trunk obesity.

Striae: present on the abdomen. Purple.

Face: moon face. Plethoric.

No muscle weakness or oedema.

Psychological disturbances.

c. Investigations:

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 111%, PCV 51%, MCHC 32%, WCC 11200, Diff. N 68%, L 28%, M 1%, B 1%, E 2%.

Electrolytes in blood: Na 141 meq/1, K 4.8 meq/1, HCO_3 23.3 meq/1, BU 12 mg./100 ml.

GPT less than 20 units/ml.

Alk.Phos. 7 K-A units/100 ml, T.T. 1 unit.

G.T.T.: F 146 mg./100 ml, ½ hr 204 mg./100 ml, 1 hr 264 mg./100 ml, 1½ hrs 246 mg./100 ml, 2 hrs 200 mg./100 ml, 2½ hrs 182 mg./100 ml, 3 hrs 160 mg./100 ml.

Urine examination: glycosuria.

Cr.cl 115 ml/min.

d. Treatment and follow up: bilateral adrenalectomy and auto-transplantation. Marked improvement of clinical condition post-operatively - glycosuria persisted.

Case No. 15 H.T. Sex: F Age: 21 yrs.

a. Symptoms: progressive increase in weight, hirsutism, amenorrhoea and depression. Duration 18 months.

b. Examination: Wt: 85.1 Kg. Ht: 158.7 cm. B.P. $\frac{125}{70}$

Skin: NAD No acne or bruising.

Hirsutism: marked. Abdominal musculine distribution.

Obesity: marked. Trunk obesity.

Striae: present on the abdomen. Purple.

Face: full. Normal genitalia.

No muscle weakness or oedema.

Psychological instability.

c. Investigations:

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 98%, PCV 45%, MCHC 32.5%, WCC 11900,

Diff. 58%, L 34%, M 5%, E 3%.

Electrolytes in blood: Na 132 meq/l, K 4.1 meq/l,

HCO_3 24.6 meq/l, BU 14 mg./100 ml.

Ca 9.9 mg./100 ml, PO_4 4.6 mg./100 ml.

d. Treatment and follow up: bilateral adrenalectomy. Marked improvement after operation of her clinical condition.

Case No. 16 E.S. Sex: F Age: 22 yrs.

a. Symptoms: increased body weight, growth of facial and abdominal hair; duration 4 years. Amenorrhoea; duration 2 years.

b. Examination: Wt: 54.2 Kg. Ht: 157.4 cm. B.P. $\frac{140}{90}$

Skin: very fine. No acne or bruising.

Hirsutism: on chin, abdomen, arms, buttock and sacrum. Male pubic distribution of hair.

Obesity: moderate. Mainly trunk.

Striae: present on upper arms and abdomen. Pale.

Face: full. No oedema or muscle weakness.

Normal genitalia.

c. Investigations:

X-ray chest: NAD

Blood picture: Hb. 96%, PCV 46%, MCHC 30%, WCC 8700,
Diff. N 61%, L 33%, M 6%.

Electrolytes in blood: Na 142 meq/l, K 3.8 meq/l,
HCO_3 27.7 meq/l, BU 10 mg./100 ml.

G.T.T.: F 88 mg./100 ml, ½ hr 140 mg./100 ml, 1 hr 94 mg./100 ml,
1½ hrs 80 mg./100 ml, 2 hrs, 82 mg./100 ml, 3 hrs 80 mg./100 ml.

d. Treatment and follow up: bilateral adrenalectomy and replacement therapy.
Marked improvement after operation of the clinical condition.

Case No. 17 C.M. Sex: F Age: 18 yrs.

a. Symptoms: progressive increase of weight since age of 8. Hirsutism started with onset of menstruation.

b. Examination: Wt: 83.3 Kg. B.P. $\frac{140}{90}$

Skin: NAD No acne or bruising.

Hirsutism: marked. Upper lip, chin, chest, back, arms and legs.

Obesity: moderate. Mainly trunk.

Striae: present on upper arms and flanks. Pink.

No muscle weakness or oedema. Normal genitalia.

c. Investigations:

ECG: NAD

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 90%, PCV 40%, platelets 320,000, WCC 5800.

Electrolytes in blood: Na 146 meq/l, K 4.1 meq/l,

HCO_3 23 meq/l, BU 16 mg./100 ml.

G.T.T.: F 88 mg./100 ml, ½ hr. 114 mg./100 ml, 1 hr 122 mg./100 ml, 1½ hrs 108 mg./100 ml, 2 hrs 83 mg./100 ml, 2½ hrs 92 mg./100 ml, 3 hrs 92 mg./100 ml, 3½ hrs 86 mg./100 ml.

III 'Cushingoid' group

Case No. 18 A.D. Sex: F Age: 19 yrs.

a. Symptoms: increase in weight, tiredness, low back pain, striae on thighs.

Duration 6 months.

b. Examination: Wt: 79.8 Kg. Ht: 157.4 cm. B.P. $\frac{130}{80}$

Skin: NAD No acne or bruising. No hirsutism.

Obesity: moderate. General but mainly trunk obesity.

Striae: present on thighs and abdomen. Purple.

Face: moon face. Normal Genitalia.

Muscle weakness: present.

No oedema.

Marked psychological instability.

c. Investigations:

ECG: NAD

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 93%, PCV 41%, MCHC 33%, WCC 4200, Diff. N 65%, L 30%, M 3%, E 2%, platelets 150,000.

Electrolytes in blood: Na 138 meq/l, K 3.7 meq/l, HCO_3 25.3 meq/l, BU 32 mg./100 ml.

Ca 11.2 mg./100 ml, PO_4 4.7 mg./100 ml.

Bili. 0.3 mg./100 ml, GPT less than 20 units/ml.

Alk.Phos. 4 K-A units/100 ml, T.T. 1 unit .

G.T.T.: F 92 mg./100 ml, ½ hr 118 mg./100 ml, 1 hr 104 mg./100 ml, 1½ hrs 106 mg./100 ml, 2 hrs 82 mg./100 ml.

Cr.cl. 123 ml/min.

d. Follow-up: patient was kept under observation. The clinical condition deteriorated after 5 months and the patient became more psychologically unbalanced.

Case No. 19 W.C. Sex: M Age: 27

a. Symptoms: progressive increase in weight since 6 years. Dyspnoea.
Excessive sweating.

b. Examination: Wt: 133.6 Kg. Ht: 180.3 cm. B.P. $\frac{180}{118}$

Skin: NAD No acne or bruising.

Obesity: marked. Mainly trunk.

Striae: present over axillae, chest, abdomen and thighs. Purple.
Duration 1 year.

Face: moon face, plethoric. No muscle weakness or oedema.

c. Investigations:

ECG NAD

X-ray skeleton: Lumbar osteoporosis.

Blood picture: Hb. 80%, PCV 59%, MCHC 31%, WCC 11700, Diff. N 75%,
L 19%, M 6%, platelets 215,000.

Electrolytes in blood: Na 138 meq/l, K 3.8 meq/l,
HCO_3 30.0 meq/l, BU 40 mg./100 ml.

Ca 9.8 mg./100 ml.

Bili. 0.5 mg./100 ml, GPT less than 20 units/ml,

Alk.Phos. 10 K-A units/100 ml. T.T. 2 units.

G.T.T.: F 62 mg./100 ml, ½hr 116 mg./100 ml, 1 hr 112 mg./100 ml,
1½ hr 66 mg./100 ml, 2 hr 40 mg./100 ml.

Cr.cl 138 ml/min.

d. Follow-up: had it not been for the fact that the caloric intake was 12,000 calories/day, a diagnosis of Cushing's syndrome would have been justified. The patient was given a dietary regimen to reduce his weight and was kept under observation.

Case No. 20 D.M. Sex: M Age: 21 yrs.

a. Symptoms: increase in weight. Dyspnoea. Oedema around the ankles.

Duration 6 years.

b. Examination: Wt: 121.8 Kg. Ht: 175.2 cm. B.P. $\frac{150}{80}$

Skin: NAD No acne or bruising.

Obesity: marked. Mostly on face, neck, abdomen and buttocks.

Striae: Present on the abdomen, chest and buttocks. Pink.

Face: full. No muscle weakness.

Slight oedema of ankles.

c. Investigations:

ECG NAD

X-ray chest: NAD X-ray skeleton: narrowing of L4-5 space

Blood picture: Hb. 100%, PCV 47%, MCHC 35%, WCC 8600, Diff. N 54%, L 30%, M 11%, E 5%, platelets 250,000.

Electrolytes in blood: Na 143 meq/l, K 4.2 meq/l, HCO_3 22.7 meq/l, BU 33 mg./100 ml.

Ca 10 mg./100 ml, PO_4 3.5 mg./100 ml.

Bili. 0.8 mg./100 ml. GPT less than 20 units/ml.

Alk.Phos. 13 K-A units/100 ml, T.T. 2 units.

G.T.T.: F 92 mg./100 ml, ½ hr 133 mg./100 ml, 1 hr 150 mg./100 ml, 1½ hrs 133 mg./100 ml, 2 hrs 92 mg./100 ml.

Cr.cl 157 ml/min.

d. Follow-up: patient was put on a reducing diet and kept under observation.

Case No. 21 T.M. Sex: M Age: 18 yrs.

a. Symptoms: sudden increase in weight with development of striae on the buttocks. Duration one month.

b. Examination: Wt: 86.3 Kg. Ht: 177.8 cm. B.P. $\frac{150}{90}$

Skin: NAD No acne, bruising or oedema.

Obesity: moderate. Mainly on the buttocks.

Striae: present on the buttocks. Red.

Face: full. No muscle weakness.

c. Investigations:

ECG NAD

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 102%, PCV 47%, MCHC 32%, WCC 5850.

Electrolytes in blood: Na 137 meq/1, K 4.4 meq/1,

HCO_3 25 meq/1, BU 2.9 mg./100 ml. Ca 9.8 mg./100ml. PO_4 2.9 mg./100ml.

Bili. 0.6 mg./100 ml, GPT 3 units,

Alk.Phos. 14 K-A units/100 ml, T.T. 2 units.

G.T.T.: F 103 mg./100 ml, ½hr 136 mg./100 ml, 1 hr 126 mg./100 ml,

1½ hrs 110 mg./100 ml, 2 hrs 106 mg./100 ml.

d. Follow-up: patient was put on a reducing diet. Reduction in weight and improvement of the clinical condition occurred.

Case No. 22 C.A. Sex: F Age: 26 yrs.

a. Symptoms: hirsutism, increase in weight and irregularity of periods.

Duration 4 years.

b. Examination: Wt: 61.1 Kg. Ht: 154.9 cm. B.P. $\frac{135}{75}$

Skin: NAD No bruising, oedema or acne.

Hirsutism: marked on face. Slightly on legs and abdomen.

Obesity: patient is plump rather than obese, especially at the abdomen.

Striae: present on the abdomen. White.

Face: full. No muscle weakness and normal genitalia.

c. Investigations:

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 88%, PCV 42%, MCHC 30.5%, WCC 10000,

Diff. N 54%, L 39%, M 4%, E 2%, B 1%.

Electrolytes in blood: Na 142 meq/l, K 4.5 meq/l,

HCO_3 27.7 meq/l, BU 15 mg./100 ml.

G.T.T.: F 78 mg./100 ml, ½ hr 90 mg./100 ml, 1 hr 108 mg./100 ml,

1½ hrs 112 mg./100 ml, 2 hrs 78 mg./100 ml, 2½ hrs 60 mg./100 ml.

d. Follow-up: patient was put on a reducing diet and kept under observation.

Case No. 23 D.S. Sex: F Age: 34 yrs.

a. Symptoms: obesity started 4 years ago, hirsutism started 8 years ago, lethargy began 11 years ago and irregular menstruation with periods of amenorrhoea started 16 years ago. There was a family history of obesity.

b. Examination: Wt: 118.1 Kg. Ht: 170 cm. B.P. $\frac{140}{90}$

Skin: NAD No acne, bruising or oedema.

Hirsutism: moderate.

Obesity: marked. General but mainly trunk obesity.

Striae: present on breasts and abdomen. Purple.

Face: full.

No muscle weakness and genitalia are normal.

c. Investigations:

ECG NAD

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 89%, PCV 46%, MCHC 29%, WCC 6600, Diff. N 78%, L 20%, M 1%, E 1%. Platelets 250,000.

Electrolytes in blood: Na 136 meq/l, K 4.8 meq/l, HCO_3 24.3 meq/l, BU 30 mg./100 ml.

Ca 8.9 mg./100 ml, PO_4 3.4 mg./100 ml,

Bili. 0.4 mg./100 ml, GPT Less than 20 units/ml.

Alk.Phos. 7 K-A units/100 ml. T.T. 2 units.

G.T.T.: F 84 mg./100 ml, ½ hr 128 mg./100 ml, 1 hr 150 mg./100 ml, 1½ hrs 118 mg./100 ml, 2 hrs 80 mg./100 ml.

Cr.cl. 105 ml./min.

d. Follow-up: patient was put on a reducing diet and kept under observation.

Case No. 24 M.K. Sex: F Age: 15 yrs.

a. Symptoms: sudden increase in weight, started three years ago, with pigmentation of areas of pressure (waist, brassier) and nipples.

b. Examination: Wt: 76.9 Kg. Ht: 158.7 cm. B.P. $\frac{115}{70}$

Skin: NAD Slight acne on face.

No bruising, oedema or hirsutism.

Obesity: moderate. Mainly trunk and upper thighs.

Striae: present on breasts, abdomen and axillae. Pinkish white.

Face: round. No muscle weakness and genitalia are normal.

c. Investigations:

Blood picture: Hb. 96%, PCV 36%.

Blood electrolytes: Na 145 meq/l, K 4.7 meq/l,

HCO_3 23.5 meq/l, BU 30 mg./100 ml.

d. Follow-up: patient was put on a reducing diet and kept under observation.

Case No. 25 D.Y. Sex: F Age: 32 yrs.

a. Symptoms: increase in weight, hirsutism of the face and oedema around the ankle. Duration 13 years.

b. Examination: Wt: 75.7 Kg. Ht: 148.5 cm. B.P. $\frac{120}{70}$

Skin: NAD No bruising or oedema but acne are present on face.

Hirsutism: present on chin, side of face, around nipples, on legs and arms. Pubic male distribution.

Obesity: moderate. Mainly trunk obesity.

Striae: present on abdomen, thighs and arms. Purple.

Face: round.

No muscle weakness and genitalia are normal.

c. Investigations:

ECG NAD

X-ray chest: NAD

Blood picture: Hb. 77%, WCC 8500.

Electrolytes in blood: Na 131 meq/1, K 4.6 meq/1, HCO_3 21 meq/1, BU 34 mg./100 ml.

Ca 11.2 mg./100 ml, PO_4 3.7 mg./100 ml.

Bili. 0.5 mg./100 ml, GPT less than 20 units/ml.

Alk.Phos. 7 K-A units/100 ml.

G.T.T.: F 75 mg./100 ml, ½hr 158 mg./100 ml, 1 hr 220 mg./100 ml, 1½ hrs 175 mg./100 ml, 2 hrs 116 mg./100 ml, 2½ hrs 90 mg./100 ml.

d. Follow-up: patient was put on a reducing diet and kept under observation.

IV Obese group

Case No. 26 A.M. Sex: M Age: 34 yrs.

a. Symptoms: obesity, breathlessness on slight or moderate exertion. Duration since childhood.

b. Examination: Wt: 152.7 Kg. Ht: 179 cm. B.P. $\frac{170}{120}$

Skin: NAD No bruising, acne, oedema or muscle weakness.

Obesity: marked. General.

Striae: present on the upper thighs. White.

Face: normal.

c. Investigations:

ECG NAD

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 95%, PCV 47%, MCHC 31.5%, WCC 7400, Diff. N 53%, L 38%, M 9%, platelets 310,000.

Electrolytes in blood: Na 144 meq/l, K 3.7 meq/l, HCO_3 27.6 meq/l, BU 8 mg./100 ml.

Ca 11.6 mg./100 ml, PO_4 4 mg./100 ml. Bili. 0.3 mg./100 ml.

Alk.Phos. 6 K-A units/100 ml. T.T. 1 unit. GPT less than 20 units/ml.

G.T.T.: F 82 mg./100 ml, ½ hr 102 mg./100 ml, 1 hr 120 mg./100 ml, 1½ hrs 80 mg./100 ml, 2 hrs 72 mg./100 ml, 2½ hrs 72 mg./100 ml, 3 hrs 70 mg./100 ml.

Cr.cl 61 ml./min.

d. Follow-up: patient was put on a reducing diet and kept under observation.

Case No. 27 R.A. Sex: M Age 12 yrs.

a. Symptoms: breathlessness on slight or moderate exertion. Of 1-2 years duration.

b. Examination: Wt: 67.4 Kg. Ht. 149.8 cm. B.P. $\frac{140}{96}$

Skin: NAD No bruising, oedema or muscle weakness.

Slight acne on face.

Obesity: moderate. Mainly trunk.

Striae: present on thighs and axillae. Red.

Face: normal.

c. Investigations:

ECG NAD

X-ray chest: NAD X-ray skeleton: slight loss of bone density (except in skull).

Blood picture: Hb. 88%, PCV 40%, MCHC 33%, WCC 3800,

Diff. N 32%, L 58%, M 10%, platelets 320,000.

Electrolytes in blood: Na 138 meq/l, K 4.5 meq/l,

HCO_3 23.8 meq/l, BU 32 mg./100 ml.

Ca 11.2 mg./100 ml, PO_4 4.8 mg./100 ml.

Bili. 0.2 mg./100 ml. GPT less than 20 units/ml.

Alk.Phos. 19 K-A units/100 ml. T.T. 1 unit.

G.T.T.: F 80 mg./100 ml, ½ hr 84 mg./100 ml, 1 hr 92 mg./100 ml,

1½ hrs 89 mg./100 ml, 2 hrs 88 mg./100 ml.

Cr.cl 108 ml/min.

d. Follow-up: patient was put on a reducing diet and kept under observation.

Case No. 28 T.A. Sex: M Age: 17 yrs.

a. Symptoms: obesity. Dyspnoea on moderate exertion. Duration 8 years.

b. Examination: Wt: 143.6 Kg. Ht. 170 cm. B.P. $\frac{140}{80}$

Skin: NAD No acne, bruising or oedema.

Obesity: marked. Chest, neck, abdomen and buttocks.

Striae: Present on thighs and abdomen. Purple.

Face: normal but slightly plethoric.

No muscle weakness.

c. Investigations:

ECG NAD

Blood picture: Hb. 95%, PCV 43%, MCHC 32%, WCC 8300, Diff. N 51%, L 32%, M 11%, E 6%, platelet 250,000.

Electrolytes in blood: Na 136 meq/l, K 3.9 meq/l, HCO_3 20 meq/l, BU 31 mg./100 ml.

Ca 9.6 mg./100 ml, PO_4 3.2 mg./100 ml.

Bili. 0.8 mg./100 ml, GPT less than 20 units/ml.

Alk.Phos. 13 K-A units/100 ml, T.T. 1 unit.

G.T.T.: F 78 mg./100 ml, ½hr 116 mg./100 ml, 1 hr 98 mg./100 ml, 1½ hrs 70 mg./100 ml, 2 hrs 64 mg./100 ml.

Cr.cl. 234 ml/min.

d. Follow-up: patient was put on a reducing diet and kept under observation.

<u>Case No. 29</u> C.S. Sex: F Age: 14 yrs.

a. Symptoms: increase in weight. Duration 3 years. Family history of obesity.

b. Examination: Wt: 96.3 Kg. Ht: 163.8 cm. B.P. $\frac{120}{80}$

Skin: NAD No bruising, acne or oedema

No hirsutism or muscle weakness.

Obesity: marked. General.

Striae: few on thighs and axillae. Red.

Face: full. Normal genitalia.

c. Investigations:

ECG NAD

X-ray skeleton: NAD

Blood picture: Hb. 98%, PCV 43%, MCHC 34%, WCC 7700, Diff. N 64%,

L 27%, M 8%, E 1%, platelets 250,000

Electrolytes in blood: Na 134 meq/l, K 4.3 meq/l,

HCO_3 24.8 meq/l, BU 27 mg./100 ml.

Ca 10.4 mg./100 ml, PO_4 3.3 mg./100 ml.

Bili. 0.6 mg./100 ml. GPT less than 20 units/ml.

Alk. Phos. 9 K-A unit/100 ml, T.T. 1 unit.

G.T.T.: F 70 mg./100 ml, ½ hr 78 mg./100 ml, 1 hr. 76 mg./100 ml,

1½ hr 76 mg./100 ml, 2 hr 78 mg./100 ml, 2½ hr 53 mg./100 ml,

3 hr 53 mg./100 ml.

Cr.cl 123 ml/min.

d. Follow-up: patient was put on a reducing diet and kept under observation.

Case No. 30 H.L. Sex: F Age: 31 yrs.

a. Symptoms: gradual increase in weight. Vague sensations of aches and dizziness. Thirst, polyuria and depression. Duration 1 year.

b. Examination: Wt: 58.2 Kg. Ht: 160 cm. B.P. $\frac{160}{94}$

Skin: rather dry. No acne, bruising or oedema. No hirsutism.

Obesity: slight. General.

Striae: nil.

Face: normal.

No muscle weakness. Normal genitalia.

c. Investigations:

ECG ?RBBB (Right bundle branch block)

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 89%, PCV 42%, MCHC 31.5%, WCC 7000, Diff. N 65%, L 30%, M 3%, E 2%, platelets 150,000.

Electrolytes in blood: Na 138 meq/l, K 4.3 meq/l, HCO_3 23 meq/l, BU 32 mg./100 ml.

Ca 10.4 mg./100 ml, PO_4 3.1 mg./100 ml.

Bili. 0.3 mg./100 ml. GPT less than 20 units/ml.

Alk.Phos. 5 K-A units/100 ml. T.T. 6 units.

G.T.T.: F 82 mg./100 ml, ½ hr 114 mg./100 ml, 1 hr 100 mg./100 ml, 1½ hr. 92 mg./100 ml, 2 hr 84 mg./100 ml.

Cr.cl 109 ml./min

d. Follow-up: patient was put on a reducing diet and kept under observation.

Case No. 31 A.I. Sex: F Age: 24 yrs.

a. Symptoms: increase in weight of 3 years duration. Lethargy, bouts of depression and irritability and irregularities of menstruation of 7 months duration. There was a family history of obesity.

b. Examination: Wt: 97 Kg. Ht: 160 cm. B.P. $\frac{110}{80}$

Skin: soft No acne, bruising or oedema.

No hirsutism or muscle weakness. Normal genitalia.

Obesity: marked. Mainly on chest and abdomen.

Striae: present on abdomen and axillae. Purple.

Face: full.

c. Investigations:

ECG NAD

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 92%, PCV 43%, MCHC 31.5%, WCC 7400, Diff. N 41%, L 53%, M 3%, E 3%, platelets 270,000.

Electrolytes in blood: Na 136 meq/1, K 4.5 meq/1, HCO_3 24.9 meq/1, BU 29 mg./100 ml.

Ca 9.5 mg./100 ml, PO_4 4.0 mg./100 ml.

Bili. 0.5 mg./100 ml. GPT less than 20 units/ml.

Alk.Phos. 5 K-A units/100 ml, T.T. 2 units.

G.T.T.: F 92 mg./100 ml, ½ hr 140 mg./100 ml, 1 hr 140 mg./100 ml, 1½ hrs 120 mg./100 ml, 2 hrs 124 mg./100 ml.

Cr.cl. 142 ml/min.

d. Follow-up: patient was put on a reducing diet and was kept under observation.

Case No. 32 C.R. Sex: F Age: 20 yrs.

a. Symptoms: increase of weight (38 Kg.) and amenorrhoea of 14 months duration. Low back pain and blackouts of 6 months duration. Irregularity of menstruation since menarche.

b. Examination: Wt: 95.9 Kg. Ht: 151.1 cm. B.P. $\frac{160}{98}$

Skin: NAD No acne, bruising or oedema.

No hirsutism.

Obesity: marked. General but mainly on trunk.

Striae: present on axillae, thighs and abdomen. Reddish.

Face: round. Normal genitalia.

No muscle weakness.

c. Investigations:

ECG NAD

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 91%, PCV 42%, MCHC 32%, WCC 6800, Diff. N 64%, L 34%, M 3%, platelets 350,000.

Electrolytes in blood: Na 142 meq/l, K 3.9 meq/l, HCO_3 27.3 meq/l, BU 20 mg./100 ml.

Ca 11.5 mg./100 ml, PO_4 3.6 mg./100 ml.

Bili. 0.2 mg./100 ml. GPT less than 20 units/ml.

Alk.Phos. 80 K-A units/100 ml, T.T. 1 unit.

G.T.T.: F 74 mg./100 ml, ½ hr 152 mg./100 ml, 1 hr 150 mg./100 ml, 1½ hrs 98 mg./100 ml, 2 hrs 68 mg./100 ml, 2½ hrs 76 mg./100 ml.

Cr.cl. 109 ml/min.

d. Follow-up: Patient was put on a reducing diet and kept under observation.

Case No. 33 M.S. Sex: F Age: 14

a. Symptoms: obesity since she was infant. Recent increase in obesity and irregular menstruation. There was a family history of obesity.

b. Examination: Wt: 92.9 Kg. Ht: 162.5 cm. B.P. $\frac{130}{90}$

Skin: NAD No bruising, oedema or acne.

No hirsutism or muscle weakness.

Obesity: marked. General.

Striae: present on abdomen and axillae. Pink.

Face: round and slightly plethoric.

Normal genitalia.

c. Investigations:

X-ray chest: NAD X-ray skeleton: NAD

Blood picture: Hb. 85%, PCV 39%, MCHC 32.5%, WCC 4200,

Diff. N 54%, L 32%, M 11%, E 2%, B 1%, platelets 185,000.

Electrolytes in blood: Na 136 meq/l, K 3.9 meq/l,

HCO_3 26 meq/l, BU 29 mg./100 ml.

Ca 10.6 mg./100 ml, PO_4 4.0 mg./100 ml.

Bili. 0.2 mg./100 ml, GPT less than 20 units/ml.

Alk.Phos. 13 K-A units/100 ml. T.T. 1 unit.

G.T.T.: F 112 mg./100 ml, ½ hr 160 mg./100 ml, 1 hr 124 mg./100 ml,

1½ hrs 132 mg./100 ml, 2 hrs 102 mg./100 ml, 2½ hrs 102 mg./100 ml,

3 hrs 100 mg./100 ml.

Cr.cl 170 ml/min.

d. Follow-up: patient was put on a reducing diet and kept under observation.

Appendix II

Abbreviations and trivial names

A. Steroids

Adrenosterone	Androst-4-ene-3,11,17-trione
Aldosterone	11β,21-dihydroxy-3,20-dioxopregn-4-ene-18-al
Allo-	5α-derivative
Androstenedione	Androst-4-ene-3,17-dione (Δ^4-androstenedione)
Androsterone	3α-hydroxy-5α-androstan-17-one
Cholesterol	Cholest-5-ene-3β-ol
Compound A (Kendall's)	11-dehydro-corticosterone 21-hydroxypregn-4-ene-3,20,11-trione
Compound B (Kendall's)	Corticosterone, Reichstein's compound H (see below)
Compound E (Kendall's)	Cortisone, Reichstein's Compound Fa (see below)
Compound F (Kendall's)	Cortisol, Reichstein's compound M (see below)
Compound S (Reichstein's)	11-deoxycortisol 17α,21-dihydroxypregn-4-ene-3,20-dione
Cortexone	DOC (see below)
Corticosterone	11β,21-dihydroxypregn-4-ene-3,20-dione
Cortisol	Hydrocortisone 11β,17α,21-trihydroxypregn-4-ene-3,20-dione
Cortisone	17α,21-dihydroxypregn-4-ene-3,11,20-trione
Cortol	
α-cortol	5β-pregnane-3α,11β,17α,20α,21-pentol
β-cortol	5β-pregnane-3α,11β,17α,20β,21-pentol
Cortolone	
α-cortolone	3α,17α,20α,21-tetrahydroxy-5β-pregnane-11-one
β-cortolone	3α,17α,20β,21-tetrahydroxy-5β-pregnane-11-one
CPR	Cortisol production rate
Dexamethasone	16α-methyl-9α-fluoro-11β,17β,21-trihydroxy-pregn-1,4-diene-3,20-dione
DHA	Dehydroepiandrosterone 3β-hydroxyandrost-5-ene-17-one
DHE	Dihydrocortisone 17α,21-dihydroxy-5β-pregnane-3α,11β,20-trione
DHF	Dihydro- cortisol 11β,17α,21-trihydroxy-5β-pregnane-3α,20-dione
DOC	Cortexone (Deoxycorticosterone) 21-hydroxypregn-4-ene3,20-dione
Epi-	3β-derivative
Estradiol	Estra-1,3,5(10),-triene-3,17β-diol
Etiocholanolone	3α-hydroxy-5β-androstan-17-one
9α-Fluorocortisol (9α-FF)	9α-fluoro-11β,17β,21-trihydroxy-pregn-1,4-diene-3,20-dione
20α-Hydroxycholesterol	Cholest-5-ene-3β,20-diol
17α-Hydroxypregnaneolone	3α,17α-dihydroxypregnane-20-one
17α-Hydroxypregnenolone	3β,17α-dihydroxypregn-5-ene-20-one
11β-Hydroxyprogesterone	11β-Hydroxypregn-4-ene-3,20-dione
17α-Hydroxyprogesterone	17α-Hydroxypregn-4-ene-3,20-dione

17-KS	17-ketosteroids (17-oxosteroids, 17-OS) C-19 adrenocortical steroids with a ketone group at C-17
17-KGS	17-ketogenic steroids (17-oxogenic steroids, 17-OGS) C-21 adrenocortical steroids with a 17α-hydroxyl group so that the C-17 side-chain may be oxidatively removed to give C-19 17-ketosteroids.
NPB cortisol	Non-protein-bound cortisol
Pregnanediol	5β-pregnane-3α,20α-diol
Pregnanetetrol	5β-pregnane-3α,11β,17α,20α-tetrol
Pregnanetriol	5β-pregnane-3α,17α,20α-triol
Δ^5-Pregnenetriol	Pregn-5-ene-3β,17α,20β-triol
Pregnanetriolone	3α,17α,20α-trihydroxy-5β-pregnane-11-one
Pregnanolone	3β-hydroxy-5β-pregnane-20-one
Pregnenolone	3β-hydroxypregn-5-ene-20-one (Δ^5-pregnenolone)
Progesterone	Pregn-4-ene-3,20-dione
P-S chromogens	Porter-Silber chromogens
THA	3α,21-dihydroxy-5β-pregnane-11,20-dione
THB	3α,11β,21-dihydroxy-5β-pregnane-20-one
TH-DOC	Tetrahydro-DOC 3α,21-dihydroxy-5β-pregnane-20-one
THE	Tetrahydrocortisone 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione
THF	Tetrahydrocortisol 3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one
THS	Tetrahydro-compound S 3α,17α,21-trihydroxy-5β-pregnane-20-one

B. Non-steroids

ACTH	Adrenocorticotrophic hormone, corticotrophin
Alk.Phos.	Alkaline phosphatase
Bili.	Bilirubin
Blue tetrazolium (BTZ)	3,3'-dianisole-bis-4,4'(3,5-diphenyl) tetrazolium chloride
B.P.	Blood pressure
BU	Blood urea
Ca	Calcium
CBG	Corticosteroid-binding globulin
Cr.cl	Creatinine clearance
cpm	counts per minute
CRF	Corticotrophin-releasing factor (s)
DDD	1,1'-dichloro-2,2'bis (p-chlorophenyl)-ethane
Diff.	Differential white cell count
Disc.Bias.	Discriminator bias volt
DPN	Diphosphopyridine nucleotide
ECG	Electrocardiogram
EHT	Extra-high-tension volt
Glucuronic acid	Glucosiduronic acid Uridine diphosphate glucuronic acid

GPT	Glutamic-pyruvic transaminase
G.T.T.	Glucose tolerance test
Hb.	Haemoglobin
HCO_3	Bicarbonate
Ht.	Height
K	Potassium
LCM	Lean cell mass
MCHC	Mean corpuscular haemoglobin concentration
MER-29	Triparanol
Na	Sodium
NAD	No abnormality detected
O,p'DDD	2,2'-bis(p-chlorophenyl)1,1'dichloroethane
PCV	Packed cell volume
PO_4	Phosphate
PVN	Paraventricular nucleus
S.A.	Specific activity
SON	Supraoptic nucleus
SU-4885 (Metopirone)	2-methyl-1,2-bis(3-pyridyl)-1-propanone
SU-8000	3-(chloro-3-methyl-2-indenyl)-pyridine
SU-9055	3-(1,2,3,4-tetrahydro-1-oxo-2-naphthyl)-pyridine
$t\frac{1}{2}$	Biological half clearance time
TIN	Tubero-infundibular nucleus
TPN	Triphosphopyridine nucleotide
T.T.	Thymol Turbidity
WCC	White cell count

N = neutrophils
L = leucocytes
M = monocytes
E = eosinophils
B = basophils

Wt.	Weight

Appendix III

Ideal weights
(according to height and frame)*

Height (in shoes) 1 inch heel for men 2 inch heel for women	Weight in pounds (in indoor clothing)		
	Small frame	Medium frame	Large frame
	MEN		
5' 2"	112-120	118-129	126-141
3"	115-123	121-133	129-144
4"	118-126	124-136	132-148
5"	121-129	127-139	135-156
6"	124-133	130-143	138-156
7"	128-137	134-147	142-161
8"	132-141	138-152	147-166
9"	136-145	142-156	151-170
10"	140-150	146-160	155-174
11"	144-154	150-165	159-179
6' 0"	148-156	154-170	164-184
1"	152-162	158-175	168-189
2"	156-167	162-180	173-194
3"	160-171	167-185	178-199
4"	164-175	172-190	182-204
	WOMEN		
4' 10"	92-98	96-107	104-119
11"	94-101	98-110	106-122
5' 0"	96-104	101-113	109-125
1"	99-107	104-116	112-128
2"	102-110	107-119	115-131
3"	105-113	110-122	118-134
4"	108-116	113-126	121-138
5"	111-119	116-130	125-142
6"	114-123	120-135	129-146
7"	118-127	124-139	133-150
8"	122-131	128-143	137-154
9"	126-135	132-147	141-158
10"	130-140	136-151	145-163
11"	134-144	140-155	149-168
6' 0"	138-148	144-159	153-173

*Prepared by the Metropolitan Life Insurance Company. Derived primarily from data on the Build and Blood Pressure Study, 1959, Society of Actuaries.

Appendix IV

Reducing dietary regimen

(1000 calories)

Breakfast

1. A choice of one of the following items:

   (a) One egg
   (b) One rasher bacon grilled crisp and drained of fat
   (c) 3 oz. white fish
   (d) 3 oz. finnan haddock with a little milk (no added fat)

2. A choice of

   (a) $1\frac{1}{2}$ oz. bread (may be toasted)
   (b) 3 squares of ryvita

3. Tea or coffee without sugar

4. Milk and butter from daily allowances (see below)

Mid-morning

A choice of one of the following items:

(a) Cup of tea with milk (from daily allowance)
(b) Cup of bovril
(c) Cup of marmite

Lunch

1. Clear soup

2. A choice of one of the following items (no added fat in cooking)

   (a) $1\frac{1}{2}$ oz. lean red meat
   (b) " " chicken
   (c) " " cold tongue
   (d) " " lean ham
   (e) 2 oz. fish
   (f) " " tripe
   (g) " " liver

3. Large helping of tomato, salad or any vegatable (except peas, beans and potatoes)

4. A piece of fresh fruit (may be stewed in water without sugar)

Tea

1. A choice of one of the following:

   (a) $1\frac{1}{2}$ oz. bread
   (b) 3 squares ryvita
   (c) 3 rich tea biscuits

2. Tea with milk (from daily allowance) no added sugar

3. Butter from daily allowance

Supper

1. A choice of either

   (a) $1\frac{1}{2}$ oz. meat (as at lunch, item 2)
   (b) One egg and $\frac{1}{2}$ oz. cheese

2. Tomato, salad or any vegetables (as at lunch, item 3)

3. A choice of one of the following:

   (a) $1\frac{1}{2}$ oz. bread
   (b) 3 water biscuits
   (c) $2\frac{1}{2}$ oz. plain boiled potatoes

4. Helping of fruit (as at lunch)

5. Tea or coffee (milk and butter from allowance)

Daily allowance of milk : 1/4 pt.
Daily allowance of butter: 3/4 oz.

N.B. 1. No fat to be used in cooking (unless from daily allowance).
2. Sweets, chocolate or sugar are not allowed.
3. The use of salad dressing, or oil in salad, is not allowed.

Summary

1. The literature concerning human adrenal cortex and its hormones and the methods of assessing adrenocortical function has been reviewed, and an account is given of the clinical conditions, Cushing's syndrome and obesity.

2. Classification of subjects, when attempting to differentiate obesity from Cushing's syndrome, has been performed for the first time as part of this study. The subjects were classified into normal, Cushing's, obese and 'Cushingoid' groups.

3. The methods used in this research included: (a) estimations of cortisol production rate, by the blood and urine techniques, using isotopically labelled cortisol, (b) estimations of urinary 17-ketosteroid excretion, (c) estimations of urinary total 17-ketogenic steroid excretion, (d) adrenocortical stimulation tests using ACTH and (e) pituitary-adrenocortical suppression test using dexamethasone.

A new technique for the simultaneous estimation of cortisol production rate, plasma cortisol and plasma-protein binding of cortisol has been developed and an <u>in vivo</u> modification of this technique has been devised and described.

4. Cortisol production rate estimations by the blood method provided the best sensitive test for assessing adrenocortical function. Ranges for the normal group under control conditions and after ACTH stimulation have been established (17.5 - 31.2 mg./24 hrs. and 216 - 268 mg./24 hrs. respectively).

The dosage of ACTH used in this research (60 I.U. intramuscularly every 12 hrs. for 3 days) was higher than any dosage previously reported. Study of the steroid excretion pattern proved that this dosage successfully established maximum stimulation of the adrenal cortex.

All/

All cases of Cushing's syndrome had higher-than-normal control levels of cortisol production rate and were hyper-responsive to ACTH stimulation.

All obese subjects, on the other hand, had normal control levels and normal or lower-than-normal response to ACTH stimulation.

5. Cortisol production rates by the urine method were the next most sensitive test for the assessment of adrenocortical function, but estimations of urinary steroid excretion, especially 17-ketosteroids, were less sensitive, and this was particularly noticeable after ACTH stimulation.

Discrepancies between the results of the various tests was noticed. It was concluded that the discrepancies between the blood and urine methods of cortisol production rate were due to the diurnal variation of adrenocortical activity in the control estimations, and to the possible role of a second compartment after ACTH stimulation. The discrepancies between the cortisol production rate tests on the one hand and the urinary steroid excretion on the other, especially after ACTH stimulation, were thought to be due to the small percentage of cortisol metabolised as steroids extractable by the latter methods.

6. Dexamethasone suppression was marked in obese and 'Cushingoid' subjects and in two patients with Cushing's syndrome but was less marked in one case of Cushing's syndrome.

7. Taking the normal individuals and the patients with Cushing's syndrome as the two "reference groups", it was possible to re-classify the patients of the 'Cushingoid' group according to the results of the various tests performed. One patient was re-classified as having Cushing's syndrome, five patients were re-classified into the obese group and two remained in the 'Cushingoid' group.

Therefore/

Therefore this group was reduced from eight to two patients. The significance of this latter group is discussed.

8. Ranges for the plasma cortisol and plasma non-protein-bound cortisol were established in the normal group (9.7 - 15.9 μg./100 ml. and 1.0 - 2.39 μg./100 ml. respectively). A case of Cushing's syndrome gave higher values, but the obese subjects gave values comparable to the normal subjects.

9. Cortisol production rate tests were applied to assess the success of the operation of bilateral adrenalectomy with auto-transplantation of a part of one adrenal into the rectus sheath of the anterior abdominal wall as a means of treatment of Cushing's syndrome without post-operative substitution therapy. Encouraging results were obtained in one of three cases. The significance of these results are discussed.

10. No correlation was found between adrenocortical function on the one hand and total body weight, height, surface area, total body lean cell mass, total body fat or body musculature on the other. When, however, the subjects who had 30 Kg. of fat or less, or whose fat was 35% of the total body weight or less, were considered separately, a correlation was found to exist between adrenocortical function and total body fat. This suggests that obesity constitutes a mechanical stress to which the adrenal cortex reacts by increasing its secretion of cortisol up to a limit; beyond this limit the adrenal cortex can no longer cope with the extra stress provided by the increased amount of fat.

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FIGURE 1

BIOSYNTHESIS AND METABOLISM OF CORTISOL

AND

THE FORMATION OF URINARY 17-KS AND TOTAL 17-KGS

$CH_3\overset{O}{\overset{\|}{C}}-S-CoA$ + $CH_3\overset{O}{\overset{\|}{C}}CH_2\overset{O}{\overset{\|}{C}}-S-CoA$ → MEVALONIC ACID (CH_2COOH, CH_3-C-OH, CH_2CH_2OH)

ACETYL-CO-A

ACETOACETYL-CO-A

MEVALONIC ACID

ISOPRENOID UNIT (CH_3-C, CH_2-, $CH \cdot CH_2-$)

SQUALENE

LANOSTEROL

ZYMOSTEROL

DESMOSTEROL

CHOLESTEROL

Δ^5-PREGNENOLONE

17α-HYDROXY-PREGNENOLONE

Δ^5-PREGNENETRIOL

PROGESTERONE

11β-HYDROXY-PROGESTERONE

DOC

ALDOSTERONE

17α-HYDROXY-PROGESTERONE

DHA

COMPOUND S

21-DEOXY-F

Δ^4-ANDROSTENEDIONE

COMPOUND B

COMPOUND A

6β-HYDROXY-CORTISOL

CORTISOL

CORTISONE

21-DEOXY-E

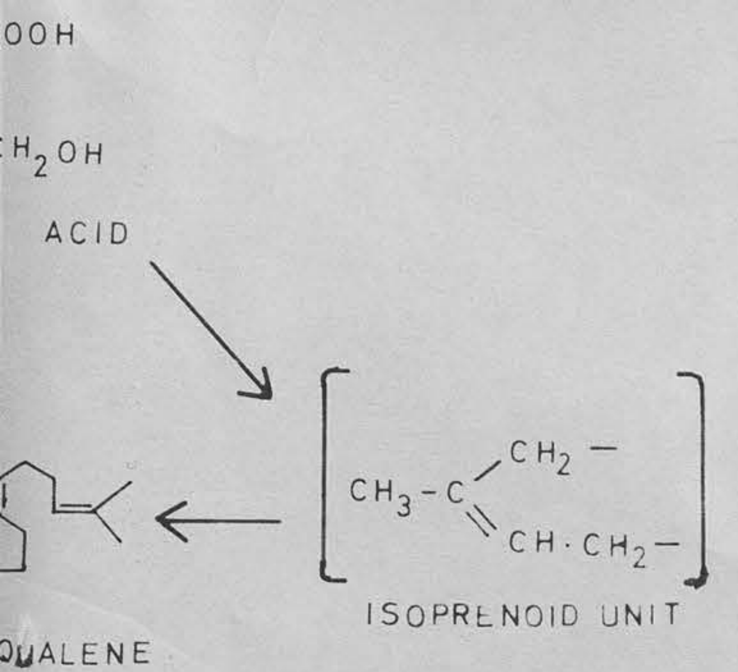
11β-HYDROXY-ANDROSTENE DIONE

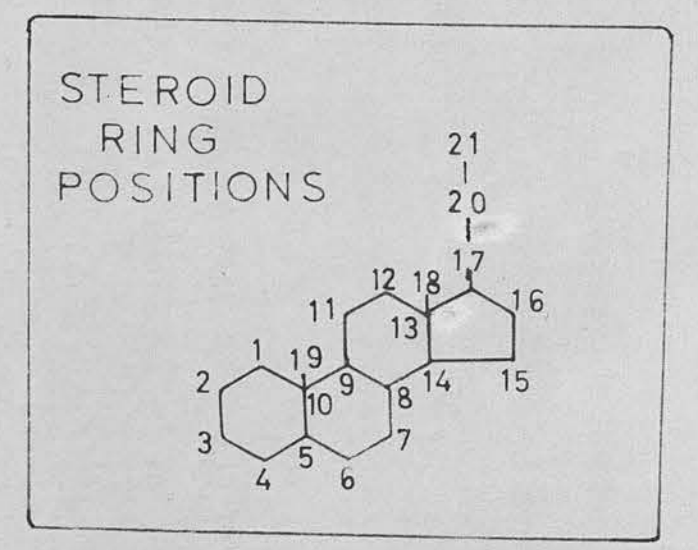
ADRENOSTERONE

Enzyme labels on arrows: 21, 11, 17, 21, 11, 11, 17, 17, 21, 11

FIGURE 1

BIOSYNTHESIS AND METABOLISM OF CORTISOL AND THE FORMATION OF URINARY 17-KS AND TOTAL 17-KGS





PRINCIPAL HYDROXYLATIONS [11] [17] [21]

BIOSYNTHETIC PATHWAYS ————

METABOLIC PATHWAYS - - - - -

URINARY 17-KS ▲

URINARY TOTAL 17-KGS ●

SMOSTEROL

CHOLESTEROL

Δ^5- PREGNENOLONE

17α-HYDROXY-PREGNENOLONE

Δ^5- PREGNENETRIOL ●

PROGESTERONE

11β-HYDROXY-PROGESTERONE

LDOSTERONE

17α-HYDROXY-PROGESTERONE ●

DHA ▲

COMPOUND S ●

21-DEOXY-F

Δ^4-ANDROSTENEDIONE ▲

OESTROGENS BIOSYNTHESIS

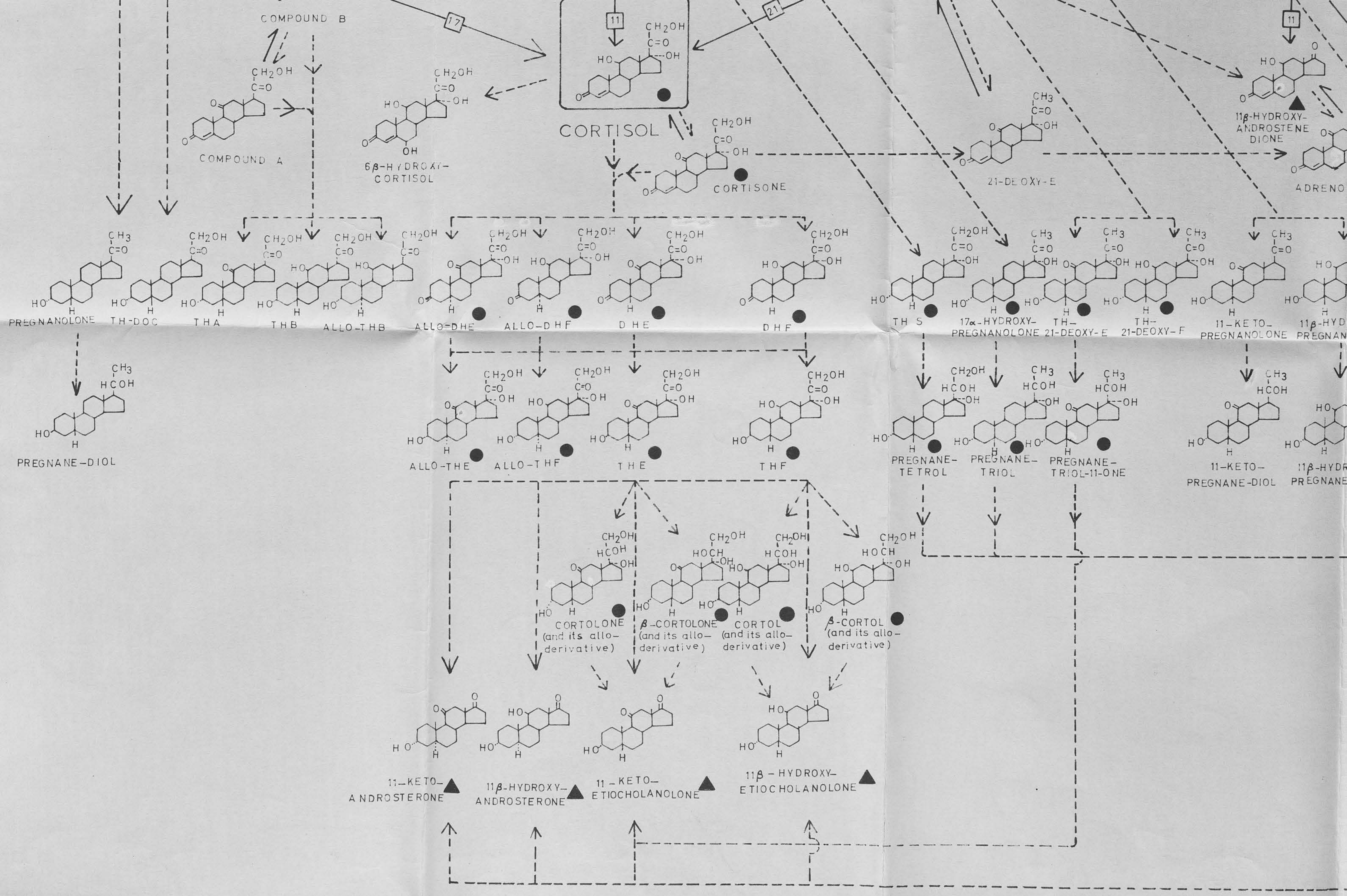
CORTISOL ●

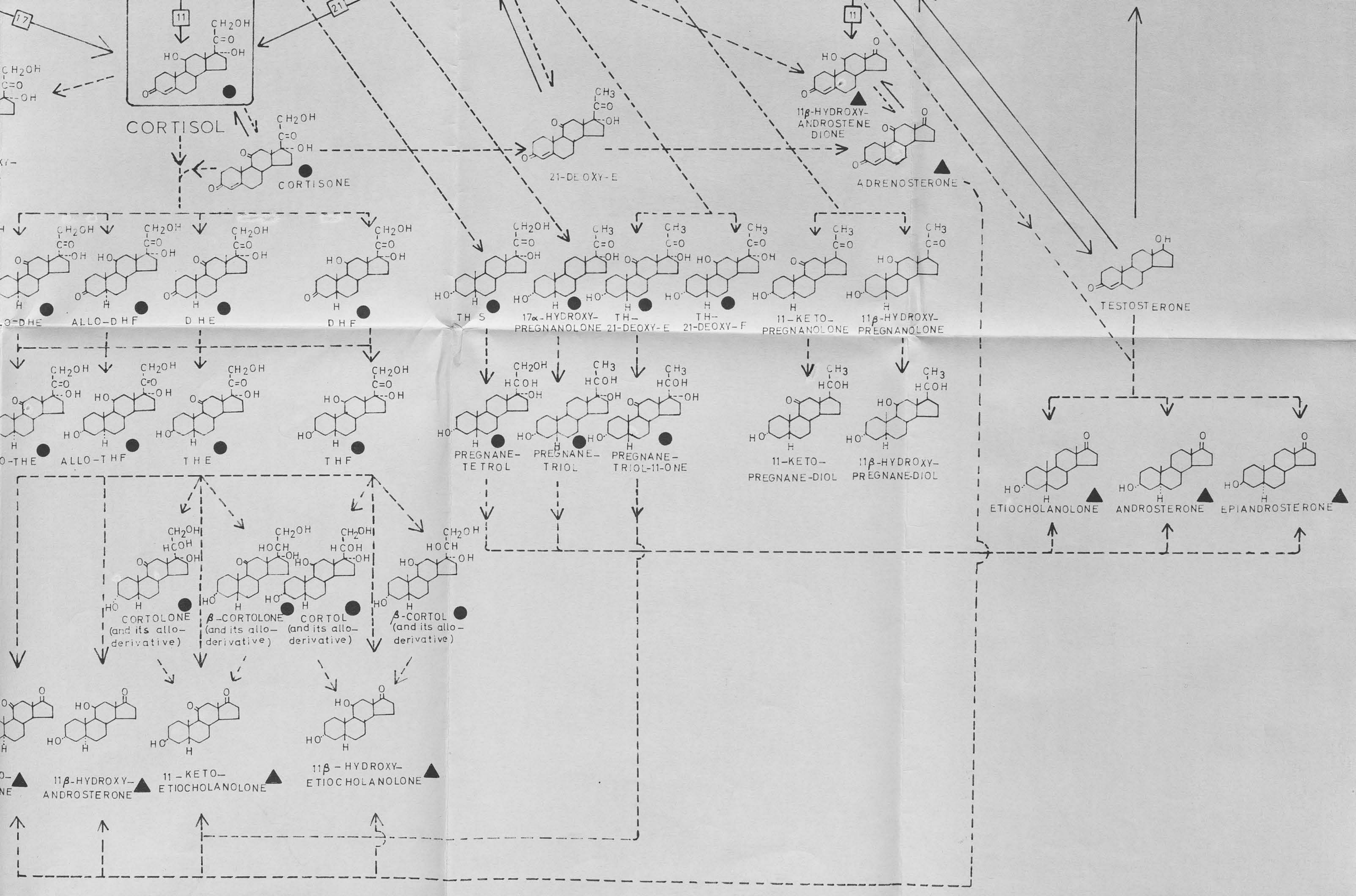
11β-HYDROXY-ANDROSTENE DIONE ▲

CORTISONE ●

21-DEOXY-E

ADRENOSTERONE ▲


COMPOUND B
COMPOUND A
6β-HYDROXY-CORTISOL
CORTISOL
CORTISONE
21-DEOXY-E
11β-HYDROXY-ANDROSTENE DIONE
ADRENO
PREGNANOLONE
TH-DOC
THA
THB
ALLO-THB
ALLO-DHE
ALLO-DHF
DHE
DHF
TH S
17α-HYDROXY-PREGNANOLONE
TH-21-DEOXY-E
TH-21-DEOXY-F
11-KETO-PREGNANOLONE
11β-HYD PREGNAN
PREGNANE-DIOL
ALLO-THE
ALLO-THF
THE
THF
PREGNANE-TETROL
PREGNANE-TRIOL
PREGNANE-TRIOL-11-ONE
11-KETO-PREGNANE-DIOL
11β-HYDR PREGNANE
CORTOLONE (and its allo-derivative)
β-CORTOLONE (and its allo-derivative)
CORTOL (and its allo-derivative)
β-CORTOL (and its allo-derivative)
11-KETO-ANDROSTERONE
11β-HYDROXY-ANDROSTERONE
11-KETO-ETIOCHOLANOLONE
11β-HYDROXY-ETIOCHOLANOLONE



CORTISOL
CORTISONE
21-DEOXY-E
11β-HYDROXY-ANDROSTENE DIONE
ADRENOSTERONE
TESTOSTERONE
ALLO-DHF
DHE
DHF
THS
17α-HYDROXY-PREGNANOLONE
TH-21-DEOXY-E
TH-21-DEOXY-F
11-KETO-PREGNANOLONE
11β-HYDROXY-PREGNANOLONE
ALLO-THF
THE
THF
PREGNANE-TETROL
PREGNANE-TRIOL
PREGNANE-TRIOL-11-ONE
11-KETO-PREGNANE-DIOL
11β-HYDROXY-PREGNANE-DIOL
ETIOCHOLANOLONE
ANDROSTERONE
EPIANDROSTERONE
CORTOLONE (and its allo-derivative)
β-CORTOLONE (and its allo-derivative)
CORTOL (and its allo-derivative)
β-CORTOL (and its allo-derivative)
11β-HYDROXY-ANDROSTERONE
11-KETO-ETIOCHOLANOLONE
11β-HYDROXY-ETIOCHOLANOLONE