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THE PATHOLOGY OF ADRENOCORTICAL HYPERFUNCTION AND THE IN VITRO BIOSYNTHESIS OF ADRENAL ANDROGENS

THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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BY

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P A R T   I

THE PATHOLOGY OF HYPERCORTICALISM

INTRODUCTION

THE STRUCTURE OF THE ADRENAL GLAND

The human adrenal glands are two small flattened structures situated retroperitoneally in the posterior part of the abdomen immediately above and to a lesser extent, in front of the upper poles of both kidneys. Each normal gland, removed surgically, weighs 4.0 g., while the weight of a normal gland at autopsy is 6.0 g. (Studzinski *et al.*, 1963). The adrenal gland is divisible into two distinct structures, an outer cortex and an inner medulla, which are different not only embryologically but also functionally. Whereas the medulla is neuroectodermal in origin, both the foetal and adult cortex arise from the coelomic mesothelium. The catecholamines, noradrenaline and adrenaline are elaborated, stored and secreted by the medulla, while the cortex forms and secretes steroid hormones, of which the most important are cortisol, corticosterone, aldosterone and dehydroepiandrosterone. No significant store of preformed steroid exists in the normal human cortex, only 20 µg. of cortisol being found per gram of adrenal tissue (O'Donnell & McCaig, 1962).

The existence of the adrenal glands was known in the sixteenth century, but it was not until the middle of the nineteenth century that significant advances occurred toward understanding its structure and possible function. In 1849, Thomas Addison

read a paper to the South London Medical Society describing "The Constitutional and Local Effects of Disease of the Suprarenal Capsules". A treatise on "Disease of the Suprarenal Capsules" followed in 1855, in which the adrenal disorder which bears his name is discussed together with his classical description of pernicious anaemia (Major, 1955). In the same year, March (quoted by Major, 1955) noted the occurrence of metastatic deposits of tumour in the suprarenal glands but that these subjects lacked evidence of Addison's disease. The first experimental demonstration of the importance of these glands was made by Brown-Séquard (1858) who noted that their removal from animals always resulted in death. It was not until 1927 that survival of bilaterally adrenalectomised animals was achieved by the administration of extracts of the adrenal cortex (Rogoff & Stewart, 1927).

In 1866, Arnold studied the reticular framework of macerated and teased sections of the human adrenal gland and described the three zones, namely the outer zona glomerulosa, the inner zona reticularis and between them, the zona fasciculata. Using histological techniques, the same zones were also described on the basis of their cellular arrangements and patterns (Gottschau, 1883). The same terminology is still in use at the present time.

Structural differences exist between the adrenal cortex of man and animals. While the rat gland possesses the three

classical zones, it contains a further lipid-free zone, the sudanophobe zone, placed below the prominent zona glomerulosa. In ruminants, by contrast, the zona fasciculata and zona reticularis form a unified lipid-sparse zone occupying the majority of the width of the cortex and lying beneath a broad zona glomerulosa. While all three zones are present in the human adrenal cortex, the zona glomerulosa is never prominent and is in fact, focal in its distribution around the periphery of the gland, being present in some areas and absent in others (Symington, 1960) (Fig. 1). Its component cells are small and are arranged in alveoli lying parallel to the surface. Some lipid is present in their cytoplasm and by histochemical techniques the DPN and TPN diaphorase systems (Dawson et al., 1961) and the Δ_5-3β-hydroxysteroid dehydrogenase systems (Wattenberg, 1958) are demonstrable. Ribonucleoprotein (RNA) and abundant mitochondria are also present (Symington, 1960).

No sharp division exists between the zona glomerulosa and the zona fasciculata and in the areas where the former is absent, the cells of the zona fasciculata extend to the capsule. This zone occupies between one half and two-thirds of the total width of the cortex and is composed of cells filled with large lipid globules, so that they appear to contain empty spaces in paraffin sections (Fig. 1). They are

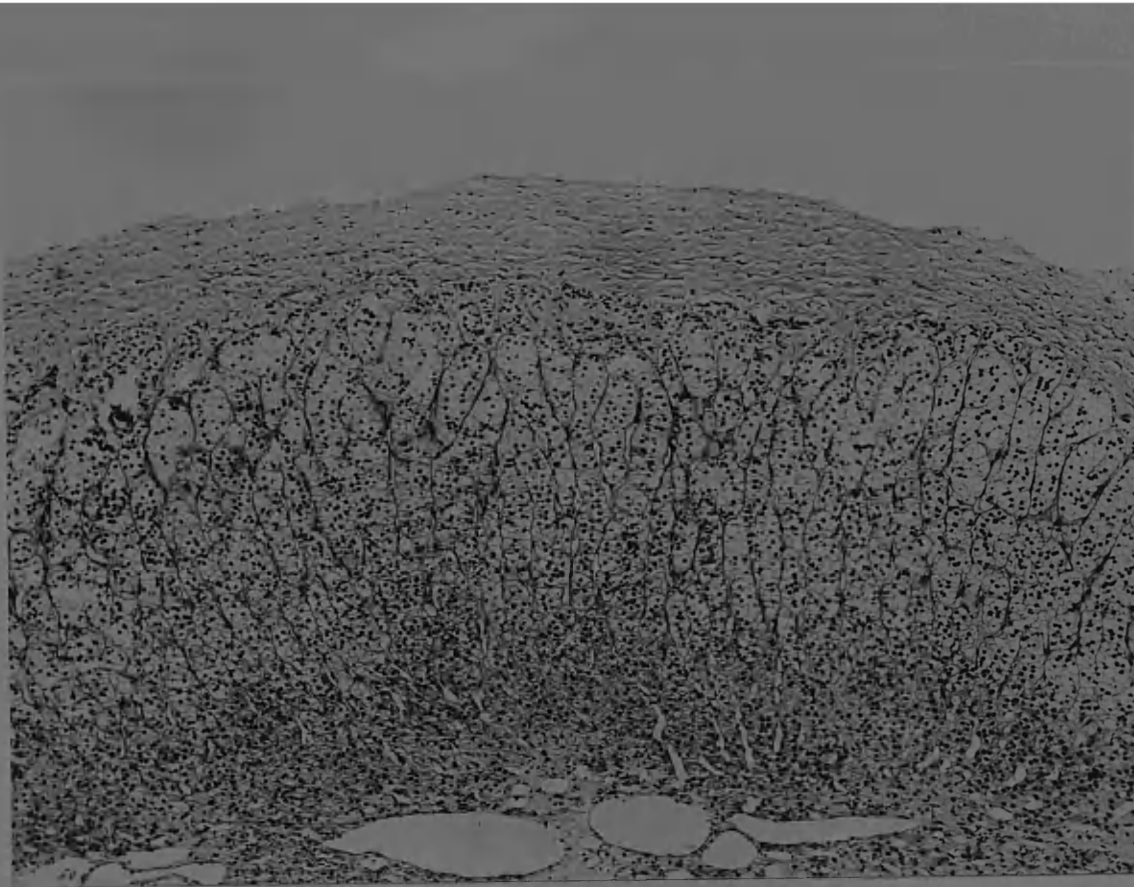


Fig. 1.- Normal human adult adrenal gland. A narrow compact cell zona reticularis occupies the inner aspect and is sharply divided from the outer clear cell zona fasciculata. The zona glomerulosa forms a patchy zone under the capsule. H & E x90.

called the "clear" cells (Symington et al., 1955). They form long cords lying in close apposition and they contain few mitochondria, little stainable RNA and are poor in the dehydrogenase enzymes of the citric acid cycle and acid and alkaline phosphatase (Symington et al., 1955). The Δ_5-3β-hydroxysteroid dehydrogenase is demonstrable in these cells, especially in the outer part of the zone (Wattenberg, 1958; Dawson et al., 1961). The change from the zona fasciculata to the zona reticularis is abrupt (Fig. 1). This latter area of the cortex is composed of small cells with an eosinophilic slightly granular cytoplasm which is poor in stainable lipid. They are referred to as the "compact" cells (Symington et al., 1955), and can be seen to contain abundant mitochondria, stainable RNA and to be rich in acid and alkaline phosphatase, and the DPN and TPN diaphorase systems. The Δ_5-3β-hydroxysteroid dehydrogenase system, by contrast, cannot be demonstrated histochemically in this zone (Wattenberg, 1958; Dawson et al., 1961) although it can be shown to be present using biochemical techniques (Grant, 1962).

THE FUNCTIONAL ZONATION OF THE ADRENAL CORTEX

The functional significance of the three separate zones has attracted much attention. Gottschau (1883) proposed the "cell migration" theory in which it was postulated that the adrenocortical

cells were formed below the capsule, migrated to the zona fasciculata where they produced their secretions and then died in the zona reticularis. Working with the cat adrenal cortex, Bennett (1940) treated sections of the gland with phenylhydrazine and noted a yellow deposit especially in the outer part of the zona fasciculata which was thought to be due to the presence of the carbonyl groups of the stored steroids. He modified Gottschau's theory and proposed that the outer zona fasciculata was the site of formation of the hormones, while the inner zona fasciculata was the site of their secretion, the zona reticularis representing the post-secretory area. This theory has become untenable due to the inability to demonstrate centripetal movement of the cortical cells (Calma & Foster, 1943) and due to the finding of a high acid and alkaline phosphatase activity in the zona reticularis of the rat gland (Yoffey, 1953; 1955).

Based upon observations in mice and rat adrenal glands and the human adrenal cortex in the adrenogenital syndrome, a "zonal" theory has been proposed by Chester Jones (1957). The zona fasciculata is thought to be the site of formation of the C_{21} corticosteroids and the zona reticularis that of the formation of adrenal androgens and oestrogens. This is founded upon a positive Vine's fuchsinophil reaction noted in the zona reticularis and thought to represent the presence of sex hormones. However,

due to the trace amounts of steroids present in the glands of both normal and virilised patients (Rogers & Williams, 1947; Bongiovanni, 1958; Neher, 1958; O'Donnell & McCaig, 1962), it is extremely unlikely that any histochemical procedure will demonstrate their presence (Symington, 1962). Similar objections can be raised to negate the histochemical staining of "ketosteroids" in the adrenal cortex (Ashbel & Seligman, 1949; Camber, 1949).

The morphological appearance of the human adrenal cortex at autopsy presented many problems in interpretation of its function. Sudden death was found to be associated with an adrenal gland which was well stocked with lipid, while in persons dying of infections, varying degrees of loss of lipid were observed (Sarason, 1943; Rogers & Williams, 1947; Ayres *et al.*, 1951; Zambeck, 1951; Stoner *et al.*, 1953). When all the lipid had been lost from the cortex, the term "exhausted" adrenal was applied to its appearance, implying loss of function (Stoner *et al.*, 1953). ACTH was also known to lead to cortical lipid depletion (O'Donnell *et al.*, 1951; Sokoloff *et al.*, 1951; Lanman, 1953). The correlation of these observations and their interpretation in terms of functional zonation of the adrenal cortex is due to the work of Symington and his colleagues (1955; 1956). Stress, such as is caused by severe burning, infections, or myocardial infarction produces a marked change in the morphology of the gland.

Lipid was lost from the cells of the zona fasciculata in a focal manner while other adjacent areas retained their lipid content. The lipid-laden cells which were converted to lipid-poor cells now resembled the cells of the zona reticularis not only morphologically but also in their enzymic, RNA and mitochondrial content. This appearance of alternating zones of clear and compact cells shown diagrammatically in Figure 2, is referred to as "focal lipid depletion" (Symington _et al_., 1955). If the stress is severe and long continued such as results from severe acute infections, the entire cortex loses its lipid content and the zona fasciculata and zona reticularis form a unified zone of compact cells referred to as "complete lipid depletion" (Symington _et al_., 1955) (Fig. 3). Children respond to stress by showing "complete lipid depletion" only (Fig. 2) and this difference may be related to the peculiar arrangement of the central adrenal venous musculature, the longitudinal muscle bundles of which may control blood flow from the cortex. They only become well developed during adult life (Dobbie & Symington, 1965). During the process of recovery from stress, lipid is initially redeposited in the zona fasciculata from within outwards so that the cells in the outer part of the zona fasciculata are the last to be restocked with lipid. This appearance is referred to as the "reversion pattern" (Sarason, 1943) (Fig. 3).

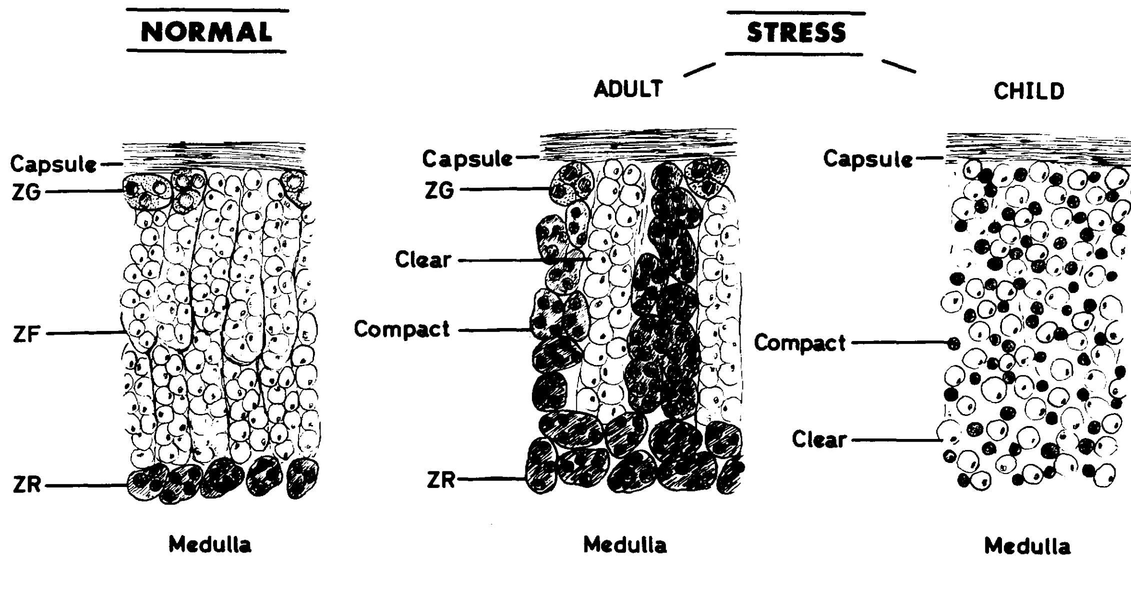


Fig. 2.- Diagramatic representation of the reaction of the human adrenal cortex to stress, with the development of focal lipid depletion in the adult and a uniform pattern of lipid depletion in the child.

REACTION

OF HUMAN ADRENAL CORTEX TO TRAUMA

COMPLETE LIPID DEPLETION REVERSION

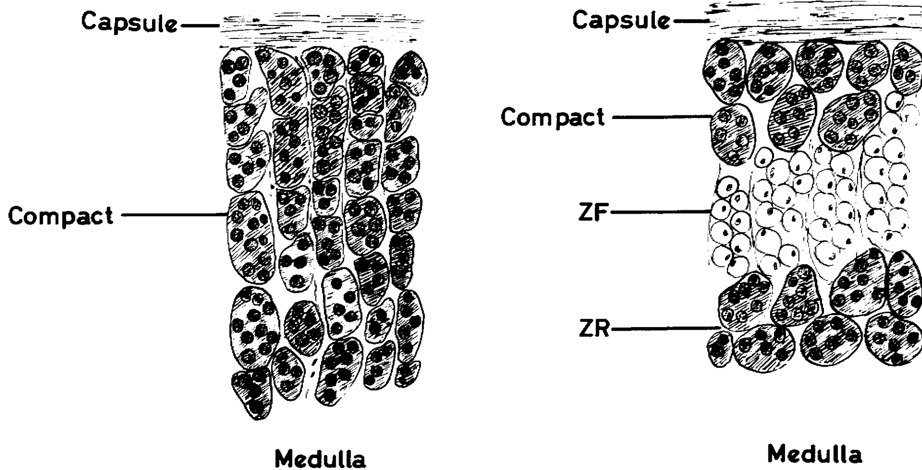


Fig. 3.- Diagramatic representation of complete lipid depletion developing in response to severe stress in the adult adrenal cortex and the reversion pattern found during recovery from stress.

These results led to the hypothesis of a unified concept of function for the zona reticularis and zona fasciculata in which the latter zone represented the site of storage of steroid hormone precursor which could be made available in times of stress through the agency of ACTH (Symington, 1960). Confirmation of this hypothesis was observed when ACTH was administered to patients undergoing therapeutic bilateral adrenalectomy for metastatic mammary carcinoma (Symington et al., 1956).

The intravenous administration of a crude preparation of ACTH led to an increase in the output of adrenal corticosteroids in the adrenal venous effluent within four minutes, yet no histological changes could be demonstrated (Grant et al., 1957). By performing the adrenalectomy in two stages, the first gland to be removed served as a control for the morphological and weight changes which occurred in the second gland following the administration of ACTH. A 110% increment in adrenal weight together with morphological and histochemical changes was observed when the crude type of ACTH was administered for four days prior to the second stage of the bilateral adrenalectomy (Studzinski et al., 1963). An alteration in the cell type, from clear to compact, occurred in the zona fasciculata from within out and in some cases, the entire cortex was changed into compact cells (Fig. 4). By using a more purified type of ACTH, the

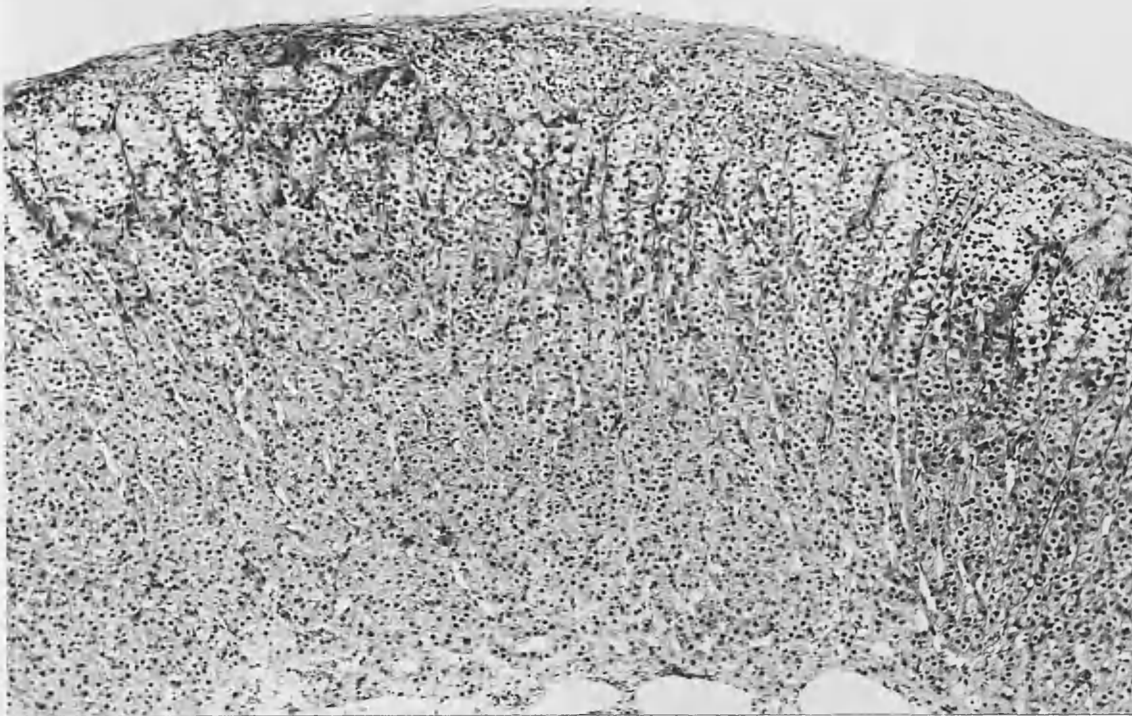


Fig. 4.- Adult human adrenal. Response to crude ACTH. There is broadening of the zona reticularis with the conversion of clear cells to compact cells, leaving only a narrow rim of clear cells in the outer aspect of the gland. H & E x95.

steroidogenic effect was found to be similar but only a slight widening of the compact cell zone occurred (Studzinski _et al_., 1963) (Fig. 5). Consequently the changes due to stress and ACTH are similar in form although different in degree depending upon the type and dose of ACTH. The change from clear to compact cells occurs initially in the cells of the zona fasciculata nearest to the zona reticularis with subsequent outward progression. It was in this zone that ^{131}I-labelled ACTH was localised in the rat by autoradiography (Sonenberg _et al_., 1951) and the highest concentrations of glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase were detected in the human and rat cortices respectively (Greenberg, 1962; Studzinski _et al_., 1962). _In vitro_ incubation of slices of the human adrenal gland obtained from the zona fasciculata and zona reticularis showed that corticosteroids were formed by both zones, but that the subsequent addition of ACTH to the medium led to an increase in steroid production principally by the cells of the zona fasciculata (Grant & Griffiths, 1962; Griffiths _et al_., 1963).

From these studies, it is evident that the zona fasciculata and zona reticularis of the human gland represent different morphological aspects of one functional zone. Both zones may contribute to the daily secretion of adrenal steroids (Griffiths _etal_., 1963) but the proportion of this secretion contributed

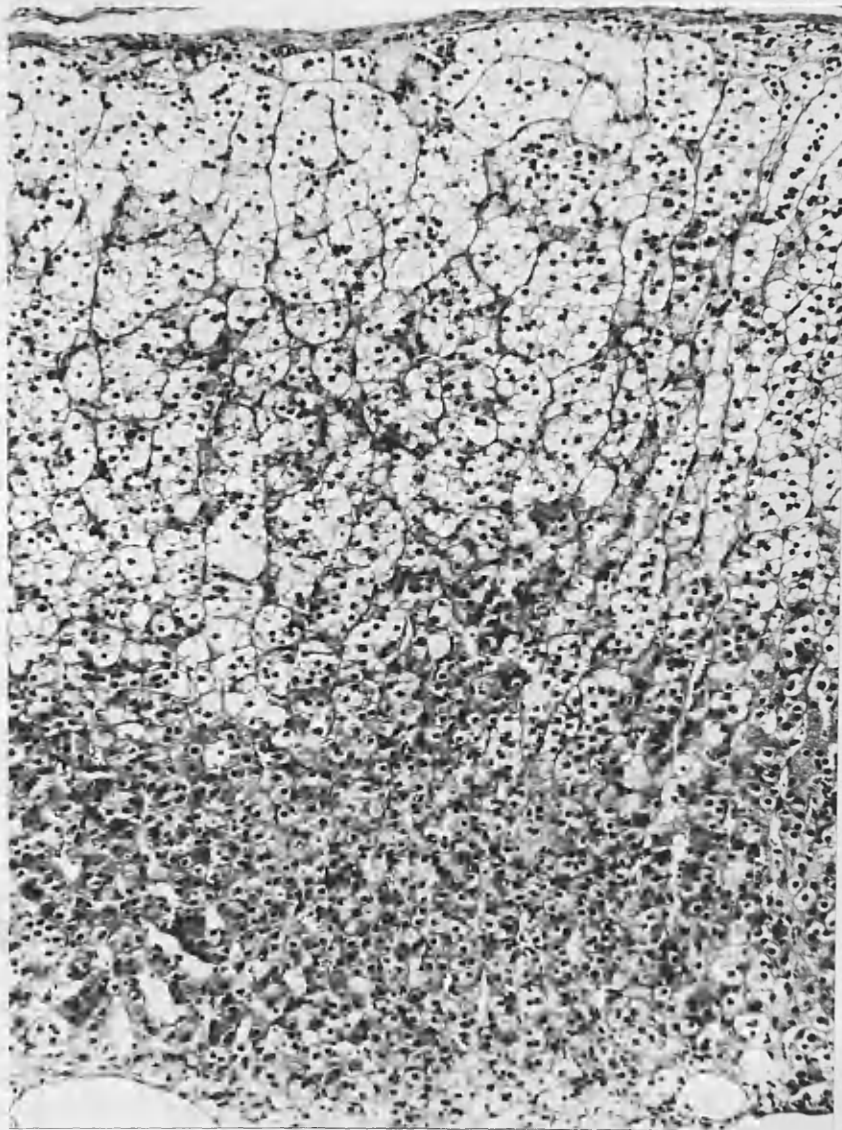


Fig. 5.- Adult human adrenal. Response to a purified form of ACTH. Slight broadening of the zona reticularis is present. The outer half of the cortex is occupied by the clear cells of the zona fasciculata. H & E x150.

by each zone remains to be elucidated. The zona glomerulosa of both animals and man is primarily concerned with the production of aldosterone (Ayres et al., 1956; Giroud et al., 1956; Ayres et al., 1958; Giroud et al., 1958), and is thus separate in its function from the remainder of the cortex.

CUSHING'S SYNDROME

In 1932, Cushing described,in a classical exposition, 16 cases of the syndrome which carries his name. All the physical findings of this disease were recorded in its first description and Cushing ascribed the cause to a pituitary basophil adenoma. Albright (1943) postulated that all the manifestations of the disease could be explained in terms of increased secretion of the adrenocortical hormones and this subsequently has been proved to be true. Between 50% to 60% of cases have been reported to be due to basophil adenomas of the pituitary (Thompson & Eisenhardt, 1943; Plotz et al., 1952) but more recently Jailer (1962) has shown that less than 10% are in fact of this aetiology. Doubt has been cast upon the type of cell involved in the pituitary tumours (Russfield et al., 1956) and chromophobe adenomas have been noted to develop following therapeutic bilateral adrenalectomy for Cushing's syndrome (Nelson et al., 1960; Salassa et al., 1959).

The association of Cushing's syndrome with acromegaly has been recorded in which an eosinophil adenoma of the pituitary was found (McCormack et al., 1951) and a few cases have been reported in which carcinomas of the adenohypophysis have been noted (Bergstrand, 1934; Cohen & Dible, 1936; Forbes, 1947; Feirnig et al., 1953; Sheldon et al., 1954 ; Haugen & Löken, 1959).

The pathology of the adrenal glands in Cushing's syndrome may be due to either tumour or bilateral hyperplasia although the gland has been recorded as being of normal weight in several instances (Crooke, 1953; Kupperman, 1953; Soffer et al., 1955; Sprague et al., 1955). Between 25% and 30% of the cases have been reported as being due to tumours (Plotz et al., 1952; Cope & Raker, 1955; Soffer et al., 1955; Sprague et al., 1955; Symington et al., 1958; Hurxthal & O'Sullivan, 1959). While benign tumours have been commoner in some series (Wilkins, 1948; Cahill & Melicow, 1950; Potasse & Higgins, 1952; Sprague et al., 1955), in others, malignant growths have predominated (Marks et al., 1940; Soffer et al., 1955). From reviews of adrenal tumours associated with Cushing's syndrome, females were found to be affected more frequently and the disease tended to occur after the age of twelve years and especially between 30 and 50 years of age (Rapaport et al., 1952; Heinbecker et al., 1957; Lipsett et al., 1963).

Bilateral hyperplasia or "normal" adrenal glands have been noted in most patients with Cushing's syndrome (Plotz et al., 1952; Cope & Raker, 1955; Soffer et al., 1955; Sprague et al., 1955; Symington et al., 1958; Hurxthal & O'Sullivan, 1959). The combined weight of the glands has varied from normal to 86 g. and 94 g. (Kovach & Kyle, 1958; Kirschner et al., 1964). Microscopically, some have been reported as normal, others as exhibiting hyperplasia of the zona fasciculata (Cohen et al., 1959; Carr et al., 1964). Loss of cortical lipid and cholesterol together with cellular atypism have been observed (Cohen et al., 1959).

Although the cause of tumours associated with Cushing's syndrome is unknown, considerable progress has been made in elucidating the aetiology of bilateral adrenocortical hyperplasia when not primarily due to a pituitary tumour. The hypothalamus was proposed as the site of the disturbance due to atrophy of the paraventricular nuclei (Heinbecker, 1944), but this has been shown to be effected by the high level of circulating corticosteroids (Castor et al., 1951). The median eminence was invoked as a possible site of the primary abnormality due to the existence of a portal venous system by which a humoral substance could pass from this site to the adenohypophysis (Harris, 1955). Such a factor has been isolated and called the corticotrophin releasing factor (CRF) (Saffran et al., 1955; Royce & Sayers, 1958) and

subsequent in vivo and in vitro studies have confirmed the ability of median eminence extracts to stimulate ACTH production by the anterior pituitary (Guillemin et al., 1957; Rumsfeld & Porter, 1959; McCann & Haberland, 1960). Consequently high peripheral plasma levels of ACTH should exist in patients with Cushing's syndrome and be responsible for the increased corticosteroid secretion by the adrenal. Attempts to demonstrate this were negative (Sydnor et al., 1953; Paris et al., 1954) but further evidence favoured the concept that the ACTH secreting mechanism was at fault. Elevated ACTH values were found in the peripheral plasma of subjects who subsequently developed pituitary tumours following therapeutic bilateral adrenalectomy for Cushing's syndrome (Liddle et al., 1959; Salassa et al., 1959; Nelson et al., 1960). In addition, resistance to the suppression of ACTH secretion was observed in patients with Cushing's syndrome due to bilateral hyperplasia (Liddle, 1960). In 1960, using different techniques, Davies and her associates found a two to threefold increase in the concentration of ACTH in the peripheral plasma in Cushing's syndrome due to adrenal hyperplasia.

Since the first report of the association of Cushing's syndrome with tumours of non-endocrine tissues (Brown, 1928) many further examples have been recorded, of which 58 were reviewed recently (Riggs & Sprague, 1961). Most were associated with

bronchogenic carcinoma, but cases with tumours of the thymus, pancreas, parotid and adrenal medulla have been recorded. ACTH or a polypeptide with similar biological activity has been demonstrated in some of these primary tumours and increased ACTH levels have also been detected in the plasma (Meador et al., 1962; Liddle et al., 1963; Hallwright et al., 1964).

THE ADRENOGENITAL SYNDROME

The adrenogenital syndrome has been a recognised entity since the days of Hippocrates (Keil, 1949), and de Crecchio (1865) reported the physical and psychological findings in a typical case. In 1817, Cooke noted its association with an adrenal tumour, while bilaterally enlarged adrenals were observed in another instance (Fibiger, 1905). Both benign and malignant tumours are associated with this syndrome, occurring more frequently in females than males and especially below the age of twelve years (Rapaport et al., 1952; Heinbecker et al., 1957). A fall in the output of urinary steroids with the administration of cortisone, cortisol or one of their synthetic analogues allows differentiation of tumours from bilateral adrenocortical hyperplasia (Wilkins et al., 1950). Bartter and his colleagues (1951) suggested that the aetiology of the cortical hyperplasia might be related to an inability to synthesise sufficient

"corticoids" for the needs of the body. This was supported by the absence of a rise in the urinary steroid output following ACTH administration (Jailer, 1953) and confirmed by the reports of low cortisol production (Kelley _et al._, 1953; Bongiovanni _et al._, 1954; Christy _et al._, 1955) and its failure to rise with ACTH (Christy _et al._, 1955). The aetiology of the low cortisol production has been shown to be due to an absolute or relative deficiency of one of three adrenocortical enzyme systems, the 21-hydroxylase (Jailer _et al._, 1955; Bongiovanni, 1958), the 11β-hydroxylase (Eberlein & Bongiovanni, 1956) or the Δ_5-3β-hydroxysteroid dehydrogenase (Bongiovanni, 1961). Both the 21-hydroxylase and the Δ_5-3β-hydroxysteroid dehydrogenase defects may be associated not only with virilism but also a tendency to salt loss when the enzyme deficiency is severe. Aldosterone secretion may be elevated, normal or low (Prader _et al._, 1955; Blizzard _et al._, 1959). Virilism and hypertension exist together when the defect is one of 11β-hydroxylation. Genetic studies have revealed that each of the enzyme disorders is inhereted as an autosomal recessive characteristic, expressing itself in the homozygous state and breeding true in any one family (Childs _et al._, 1956).

Due to the negative feed-back mechanism exerted by cortisol upon the adenophypophysis, this disease with its low cortisol

production is associated with elevated levels of blood ACTH (Sydnor *et al.*, 1953) which are of a higher order than those found in Cushing's syndrome due to bilateral adrenocortical hyperplasia (Hamilton & Brush, 1964). The increased ACTH production leads to an increment in cortisol production, if a relative deficiency only of the particular enzyme at fault is present, by causing adrenocortical hyperplasia, but this is achieved at great metabolic cost due to the increased production of other steroids especially the androgens. The weight of the adrenal glands is always increased irrespective of age (Lowenthal *et al.*, 1958; Landing & Gold, 1951; Scherz & Geppert, 1958). Contradictory reports with regard to the histopathology have been published. Blackman (1946) noted hyperplasia of the zona reticularis, and that this was progressive with increasing duration of the disease and advancing age of the patiant. The hyperplasia was considered to be due to the presence of eosinophilic "compact" cells which "encroached" upon the zona fasciculata with a resultant decrease in its volume. Those "compact" cells werethought to be derived from the foetal cortex. On the contrary, hyperplasia of the zona fasciculata (Tonutti *et al.*, 1961) and hyperplasia of the zona reticularis with a normal zona fasciculata have been recorded (Seelen, 1960). The absence of the zona glomerulosa has been observed in cases

associated with salt loss (Bongiovanni & Root, 1963).

A further histological variety of congenital adrenal hyperplasia due to an inability of the gland to convert cholesterol to pregnenolone has been described (Prader & Gurtner, 1955) in which the entire cortex was overstocked with lipid and cholesterol.

A particular variety of the adrenogenital syndrome causes feminization in the male. While this syndrome may be associated with non-tumourous adrenocortical hyperfunction (Perloff & Hadd, 1957; Gabrilove, 1958; Donald, 1961; Decourt & Guinet, 1962; Kupperman, 1963), the feminizing adrenogenital disorder is more commonly due to a tumour. Since the first reported cases (Bittorf, 1919; Parkes-Weber, 1926) 52 cases have been recorded and reviewed (Gabrilove <u>et al</u>., 1965) of which 30 were proved to be malignant by finding metastases. They occur most often between the ages of 25 and 50 years and have been proved, by <u>in vitro</u> incubation studies, to form oestrogens from suitable precursors (Baggett <u>et al</u>., 1959; West <u>et al</u>., 1964 ; Gabrilove <u>et al</u>., 1965). Of great interest are the reports of the coexistence of Cushing's syndrome and feminization in two patients (Picard <u>et al</u>., 1952; Gabrilove <u>et al</u>., 1965), as this finding lends support to the unitary concept of adrenocortical function expressed by Symington (1960; 1962).

CONN'S SYNDROME

The fourth and final clinical syndrome associated with adrenocortical hyperfunction is primary hyperaldosteronism. This entity was first described by Foye and Feichtmeir (1955) and its aetiology identified by Conn (1955), although a similar syndrome associated with mineralocorticoid excess had been recorded previously in dogs (Ferrebee et al., 1941). Most, but not all, cases are associated with a demonstrable hypersecretion of aldosterone (Conn, 1955; 1960). In vitro incubation studies with tumours causing this syndrome have revealed that they are capable of forming other corticosteroids apart from aldosterone (Bailey et al., 1960; Brode et al., 1962; Fazekas & Webb, 1965). On this account together with the fact that not all cases show an elevated secretion of aldosterone, Conn's syndrome may be a better name for this disease. When malignant tumours cause this syndrome, a rise in the 17-ketosteroids 17-hydroxysteroids or 17-ketogenic steroids can be detected (Foye & Feichtmeir, 1955; Brooks et al., 1957). The syndrome is most commonly due to a benign tumour of the adrenal cortex (Conn, 1963) indistinguishable from the "non-functioning" cortical adenoma found at autopsy (Biglieri & Forsham, 1961). They occur more commonly in females than males especially between 30 and 50 years (Conn, 1963). They are most frequently small

in size and single although multiple tumours have been recorded (Conn, 1963). Bilateral adrenocortical hyperplasia, affecting the zona glomerulosa, the known site of aldosterone formation (Ayres et al., 1956; Giroud et al., 1956; Siebenmann, 1959) is rarely the basic pathological cause. It occurs predominantly in male children and is often associated with malignant hypertension. A congenital abnormality of the renal juxtaglomerular apparatus has been suggested as the aetiology of the increased aldosterone secretion (Conn & Conn, 1961; Conn et al., 1964). Although bilateral hyperplasia of the zona glomerulosa is seen in such cases (Symington & Jeffries, 1962), its association with ostensibly normal glands has also been recorded (Holten & Petersen, 1956; Bartter & Biglieri, 1958; Conn & Conn, 1961).

From the examination of hyperplastic and neoplastic adrenal glands associated with hypercorticalism, Symington and his colleagues (Symington et al., 1958; Symington & Jeffries, 1962; Symington, 1961-62) have been able to show that the alterations which occur in the adrenal cortex in association with its various diseases, accord with their views of a unitary functional concept for the zona reticularis and zona fasciculata. This present study of 127 lesions of the adrenal cortex associated with

hypercorticalism (Table I) was undertaken with a view to assessing whether all the adrenal abnormalities found after the initial reports accorded with the concept previously discussed. Although a considerable clinical overlap may be apparent in some cases, the pathological lesions, either hyperplasia or neoplasia, have been classified according to the predominant clinical signs as Cushing's syndrome, Conn's syndrome or the adrenogenital syndrome, including feminization.

TABLE I

ANALYSIS OF 122 CASES OF

ADRENOCORTICAL HYPERFUNCTION

Disease	Number of cases	Adrenal Pathology		
		Bilateral Hyperplasia	Adenoma	Carcinoma
Cushing's Syndrome	80	69	5	6
Conn's Syndrome	23	4	17	2
Adrenogenital Syndrome (virilism)	17	7	6	4
Adrenogenital Syndrome (Feminization)	2	-	-	2

MATERIAL AND METHODS

PREPARATORY TECHNIQUES

The histological investigations were performed on material obtained either at the time of surgery or at autopsy. The surgically removed suprarenal glands or tumours were placed in polythene bags and kept on ice in a vacuum flask during transit to the laboratory. Of necessity, some of the lesions were kept on ice for periods of up to 24 hours when the operation was performed in areas of Great Britain other than Glasgow.

The adrenal glands and tumours were trimmed free of adherent tissues and weighed. Tumours were separated from the associated atrophic gland, when possible, prior to weighing. Post-mortem lesions were treated in a similar fashion following their removal.

Complete transverse sections were selected from the head, body and tail of each normal, hyperplastic or atrophic gland (Dobbie & Symington, 1965). Representative portions of each tumour were obtained from its capsular, peripheral and central parts. Areas showing evidence of haemorrhage, necrosis, cyst formation or calcification were also included when observed.

HISTOLOGICAL AND HISTOCHEMICAL TECHNIQUES

Some of the selected portions of tissue were fixed in

10% neutral formalin and subsequently embedded in paraffin or gelatine. Paraffin embedded material was sectioned at 6μ and stained with haematoxylin and eosin for general morphological purposes, or by Brachet's method for ribonucleoprotein (RNA) (Brachet, 1953). Lipid was demonstrated in gelatine embedded material sectioned at 10μ and stained by haematoxylin and Sudan IV. Paraffin embedded tissue, previously fixed in a chrome dichromate solution, pH 5.4, containing 2% osmic acid, was cut and stained by Altman's aniline acid fuchsin method for mitochondria (Culling, 1963).

The remaining portions of tissue were used to study three enzyme systems. The distribution of acid and alkaline phosphatase was demonstrated using the techniques of Rutenburg and Seligman (1955) and Burgos and his colleagues (1955) respectively and the Δ_5-3β-hydroxysteroid dehydrogenase by the method of Wattenberg (1958).

THE PATHOLOGY OF CUSHING'S SYNDROME

Cushing's syndrome accounted for 80 of the total 122 cases of adrenocortical hyperfunction composing this series. Sixty-nine of these cases were due to bilateral adrenocortical hyperplasia and 11 were caused by tumours. When the present series was totalled with the reports of Plotz (1952), Sprague (1955), and Soffer (1961) and their associates, non-tumourous involvement of the adrenal glands accounted for 74.6% of 307 cases, with benign and malignant tumours forming the remainder in almost equal proportions (Table II).

BILATERAL ADRENOCORTICAL HYPERPLASIA

A detailed analysis of the 69 cases of bilateral hyperplasia is shown in Table III. While bilateral hyperplasia was the commonest entity, evidence of adenomatous hyperplasia was noted in 10 patients and in a further 8, Cushing's syndrome and bronchogenic carcinoma were found in association with one another. Irrespective of the type of hyperplasia, 73% of the cases occurred in females and the highest age incidence was between 20 and 40 years. The youngest patient was aged 3 months and only one further case occurred below the age of 10 years. This accords with the suggestion that bilateral hyperplasia is an uncommon cause of Cushing's syndrome in children. Only a

TABLE II

CUSHING'S SYNDROME

Incidence of Pathological Lesions

Source	Number of Cases	Adrenal Pathology		
		"Normal" and Hyperplastic Glands	Adenomas	Carcinomas
Plotz, Knowlton & Ragan, 1952	94	67	11	16
Sprague, Randall, Salassa, Scholtz, Priestly, Walters & Bulbulian, 1955	88	69	14	5
Soffer, Iannaccone & Gabrilove, 1961	45	24	8	13
Present series	80	69	5	6
Total	307	229	38	40
% Incidence	-	74.6	12.4	13.0

TABLE III

CUSHING'S SYNDROME

Bilateral Adrenocortical Hyperplasia

Analysis of 69 Cases

Pathological Lesion	Number of Cases
Hyperplasia	50
Hyperplasia with tuberculosis	1
Hyperplasia with bronchogenic carcinoma	8
Adenomatous hyperplasia	10

few cases have been recorded (Chute et al., 1949; Hubble & Illingworth, 1957; Goldblatt & Snaith, 1958; Silver & Ginsburgh, 1960; Soffer et al., 1961; Thursby-Pelham & Crowe, 1961; Perlmutter, 1962; O'Bryan et al., 1964). As one might expect, almost all of the cases of Cushing's syndrome associated with bronchogenic carcinoma occurred in patients over 40 years of age.

The weights of the adrenal glands removed surgically from 50 patients, are recorded in Table IV. In 39 cases, the adrenal glands weighed less than 10 g. When the recorded weights in this series were summated with those of Sprague and his colleagues (1955), in 93 of the 119 cases, the glands weighed less than 10 g. By separating the hyperplastic glands into two groups, namely bilateral hyperplasia and bilateral adenomatous hyperplasia, distinct differences in the weights of the gland were observed (Table V). Thirty-six of the 42 glands showing only bilateral hyperplasia weighed less than 10 g., while 5 of the 8 glands exhibiting bilateral adenomatous hyperplasia weighed more than 12 g.

Previous workers have reported the finding at operation of adrenal glands of normal weight in association with Cushing's syndrome (Plotz et al., 1952; Cope & Raker, 1955; Soffer et al., 1955; Sprague et al., 1955; Hurxthal & O'Sullivan, 1959;

TABLE IV

CUSHING'S SYNDROME

Bilateral Adrenocortical Hyperplasia

Weights of Adrenal Glands Removed at Operation

Source	Number of cases	Weight of Adrenal Glands (g.)			
		8	10	12	12
Sprague, Randall, Salassa, Scholtz, Priestly, Walters & Bulbulian, 1955	69	34	20	8	7
Present series	50	29	10	4	7
Total	119	63	30	12	14

TABLE V

CUSHING'S SYNDROME

Weights of Adrenal Glands Removed at Operation

Adrenal Pathology	Number of Cases	Weight of Adrenal Glands (g.)					
		< 5	< 6	< 8	< 10	< 12	> 12
Bilateral hyperplasia	42	3†	4	20	9	4	2
Bilateral adenomatous hyperplasia	8	1*	-	1	1	-	5

† Two occurred in children
* Occurred in a 3 month old child

Soffer et al., 1961). In these studies, the weight of the normal adrenal gland was adjudged to be 6.0 g. with an upper limit of 8.0 g. While these figures are true for autopsy specimens, the weight of a normal adrenal gland removed surgically without prior ACTH or steroid therapy, is 4.0 g. with an upper limit of 6.0 g. (Studzinski et al., 1963). Consequently, of the bilateral hyperplastic glands in this series, only seven can be considered to fall within the normal range. These have been classified in detail (Table VI). Three patients are children of eleven years of age or younger. While this may explain the occurrence of glands of normal adult weight in those cases, the existence in adults of such glands is apparent. However, their weights are outwith the narrow normal range of 4.01 ± 0.02 g. found by Studzinski and his colleagues (1963).

The concomitant occurrence of bronchogenic carcinoma and Cushing's syndrome was observed in eight patients (Table VII), in one of whom (Case 8) none of the clinical stigmata of the disease was noted. Its urinary steroid biochemical abnormalities and a metabolic alkalosis were present. In sharp contradistinction to the sex incidence of Cushing's syndrome due to bilateral hyperplasia in general, seven of the eight patients were males. Apart from Cases 2 and 4 (Table VII), the glands were all examined at autopsy. In every instance, their weights were above

TABLE VI

CUSHING'S SYNDROME

Adrenal Gland of "Normal" Weight

Case Number	Age Years	Sex	Weight in g.		Comment
			Left Adrenal	Right Adrenal	
1	25	Female	4.75	2.96	Right gland incomplete
2	11	Male	5.17	5.36	-
3	11	Male	5.0	5.60	-
4	15	Female	5.81	5.69	-
5	-*	Female	5.95	5.35	-
6	10	Male	4.0	4.60	-
7	31	Male	5.0	4.90	-

* Details not available

TABLE VII

CUSHING'S SYNDROME

Non-endocrine Malignant Tumours in Association with Cushing's Syndrome

Case Number	Age Years	Sex	Weight of Adrenal Glands (g.) at Autopsy	
			Left	Right
1	53	M	12.60	11.30
2	46	M	11.70*	10.80*
3	41	M	19.0	15.60
4	-≠	M	19.10*	16.50*
5	49	F	30.0⌿	
6	-≠	M	32.0⌿	
7	40	M	34.0⌿	
8	60	M	8.0	13.0
9	38	M	7.60*	11.0

* Operation specimen
⌿ Weight of combined adrenal glands
≠ Details not available

normal and in seven cases, the increment was severalfold approximating to the weight of adrenal glands found in the adrenogenital syndrome due to congenital adrenal hyperplasia. The last case (Case 9, Table VII) is an example of the association of Cushing's syndrome and a malignant argentaffin tumour of the stomach. The difference in weights of the two adrenal glands found in this patient was due to the left gland being an operation specimen, while the right was weighed at autopsy.

Bilateral adenomatous hyperplasia was observed in the glands removed from 10 patients, of which 8 underwent therapeutic adrenalectomy (Cases 1-8, Table VIII). All but one were female. The age incidence ranged from 3 months to 47 years and without exception the adrenal glands were heavier than normal for the age of the patients and most were markedly increased in weight. Cases 9-12 are four previously reported instances of bilateral adenomatous hyperplasia (Mellinger & Smith, 1956; Mosier et al., 1960; Silverman et al., 1963; Kirschner et al., 1964). Mellinger and Smith (1956) and Kirschner and his colleagues (1964) have suggested that this type of pathological change occurs in patients who have suffered from Cushing's syndrome for many years. This is not apparent in the present series, especially with regard to Case 8 (Table VIII). This infant was of normal weight and appearance at birth, but by the age of 3 months, examination of

TABLE VIII

CUSHING'S SYNDROME

Bilateral Adenomatous Hyperplasia: Adrenal Glands Removed at Operation

Case Number	Sex	Age Years	Duration of the Disease (years)	Weight of Adrenal Glands g.		Response to ACTH Infusion	Response to Dexamethasone Administration	Authors
				Left	Right			
1	F	-≠	- ≠	14.0	14.1	Not performed	Not performed	Present series
2	F	42	- ≠	-	13.2	Not performed	Not performed	Present series
3	M	47	3-4	17.50	10.0	Not performed	Not performed	Present series
4	F	29	Over 2	8.30	8.80	No response	No suppression	Present series
5	F	27	2	6.76	7.70	Exaggerated response	Suppression	Present series
6	F	41	4	14.73	10.0	Exaggerated response	Minimal suppression	Present series
7	F	40	2	20.97	22.97	Exaggerated response	No suppression	Present series
8	F	3/12	2-3/12	1.0	3.0	Not performed	Not performed	Present series
9	F	48	30	11.0	6.0	Exaggerated response	Not performed	Mellinger & Smith, 1956
10	F	15	1	16.20⌿		No response	No suppression*	Mosier, Flynn, Will & Turner, 1960
11	M	30	4	18.0	9.0	Exaggerated response	No suppression	Silverman, Marnell, Sholiton, & Werk, 1963
12	F	40	11	44.0	50.0	Exaggerated response	No suppression	Kirschner, Powell & Lipsett, 1964

≠ Details not available
⌿ Weight of both glands
* 9α-fluorohydrocortisone

both glands after surgical removal revealed evidence of adenomatous hyperplasia. Of interest is the failure of suppression of adrenocortical secretion with dexamethasone (Cases 4, 6, 7, 10, 11 & 12; Table VIII) This same response is found in cases of simple cortical tumour. It is therefore important to establish the precise pathological diagnosis as a bilateral adrenalectomy is necessary. This operation is curative in cases of adenomatous hyperplasia.

The right and left adrenal glands usually correspond closely in weight (Studzinski et al., 1963). Of the 34 cases of bilateral non-tumourous involvement of the adrenal glands in which both adrenals were available for study, 23 pairs differed in weight by less than 1.0 g. and 5 further pairs by less than 2 g. (Table IX). Of the remaining six, discrepancy was due to adenomatous hyperplasia in three, intracortical calcification of unknown aetiology or an incomplete gland available for study in a further two.

Three points of importance emerge from a study of the weights of the adrenal glands associated with Cushing's syndrome. First, the weight of glands in which bilateral hyperplasia is found is most often less than 10 g. Secondly, if the glands weigh more than 10 g., the pathological change may be either adenomatous hyperplasia or hyperplasia associated with bronchogenic

TABLE IX

CUSHING'S SYNDROME

Weights of Both Hyperplastic Glands Removed at Operation in 34 Cases

Weight Difference less than	Number of Cases	Pathological Lesion
1.0 g.	23	-
2.0 g.	5	Adenomatous hyperplasia (2 cases) Hyperplasia (3 cases)
3.0 g.	2	Hyperplasia in association with bronchogenic carcinoma. Intracortical calcification
4.0 g.	1	Hyperplasia - one gland incomplete
5.0 g.	2	Adenomatous hyperplasia Hyperplasia in association with bronchogenic carcinoma
8.0 g.	1	Adenomatous hyperplasia

carcinoma. Finally, glands of normal weight (less than 6.0 g.) are extremely rare in adults suffering from Cushing's syndrome.

The hyperplastic gland in Cushing's syndrome is larger than normal, has rounded prominent edges and is yellow in colour. Where the gland is normal in weight, however, the macroscopic appearance is also normal. In cases of adenomatous hyperplasia, one or more prominent yellow nodules are noted on the surface of the gland, the remainder of which possesses a typical hyperplastic appearance. Occasionally, the nodules may be multiple or even wholly intracortical.

On section, the substance of the cortex consists of two distinct layers of similar proportions, the inner being brown in colour while the outer is yellow. The cut surface of adrenal glands found at autopsy presents a brown appearance extending outwards to, or almost to, the outer aspect of the glands. A similar appearance, often even broader in extent, is noted in the cortex.of those cases of Cushing's syndrome found in association with bronchogenic carcinoma.

The classical microscopic pattern of hyperplastic glands found at operation is shown in figure 6. The inner brown layer noted on gross examination corresponds to a prominent and broadened <u>compact cell</u> zona reticularis, while the outer yellow layer is caused by the <u>clear cells</u> of the zona fasciculata.

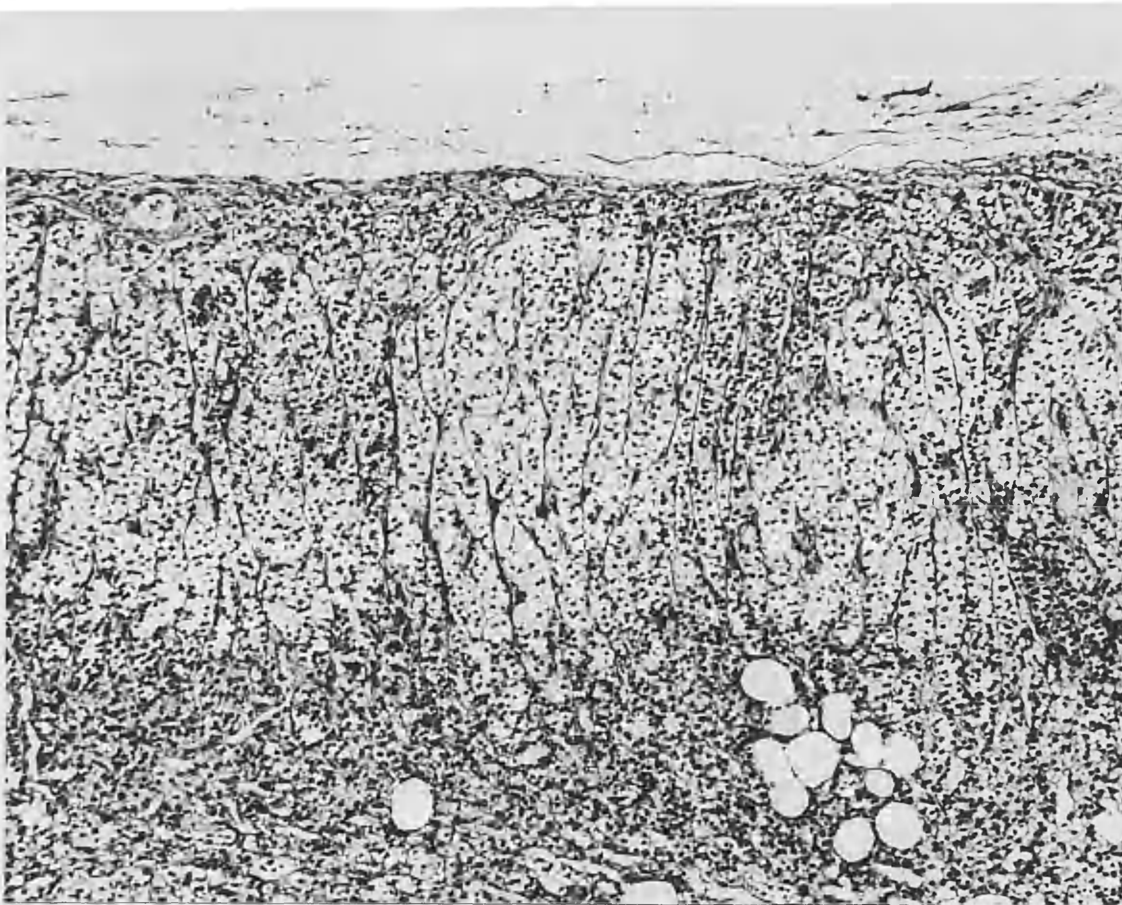


Fig. 6.- Cushing's syndrome - bilateral hyperplasia. A broad compact cell zona reticularis is present and an undulating border separates it from the zona fasciculata. Large lipid containing spaces are noted in the zona reticularis. H & E x100.

The compact cells extend to one-third to one-half of the breadth of the cortex abutting abruptly upon the overlying clear cells. The dividing line between the two zones is undulating so that clear cells may extend deep into the cortex in one area and in another, the compact cells may reach out toward the capsule (Fig. 6). The presence and distribution of RNA, mitochondria, acid and alkaline phosphatase and the Δ_5-3β-hydroxysteroid dehydrogenase correspond to the findings in normal clear and compact cells. Not only hyperplasia but also hypertrophy can affect the clear and compact cells more especially in glands which are over 12 g. in weight and which are associated with neither adenomatous hyperplasia nor bronchogenic carcinoma. Prominent nuclear pleomorphism can be seen in some of the compact cells in this type of gland (Fig. 7).

Not infrequently, large adipose spaces occur in the hyperplastic zona reticularis or at its junction with the zona fasciculata (Fig. 6). In two patients, in both of whom remission of their disease occurred, adipose tissue was found to replace the zona reticularis almost in its entirety. Two further patients were given courses of the drug o,p-DDD with subsequent temporary remission of the syndrome. Following relapse, adrenalectomy was performed and the inner aspect of the adrenal cortex was partly replaced by lipid containing spaces. The

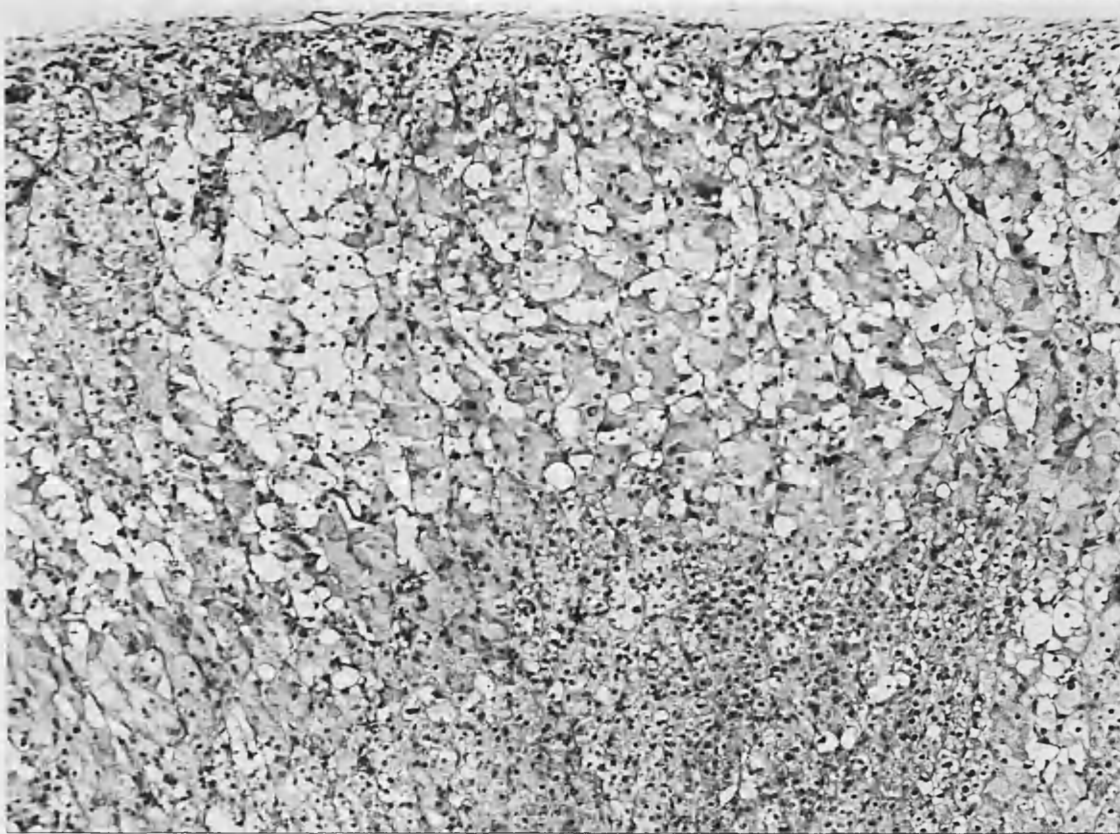


Fig. 7.- Cushing's syndrome - bilateral hyperplasia. A broad zona reticularis is present from which columns of hypertrophied compact cells pass outward toward the capsule. Foci of large clear cells remain between the compact cell columns. H & E x100.

remainder of the cortex was composed of compact cells in some of which degenerative changes were present. Only the most peripheral part of the cortex contained clear cells (Fig. 8).

The microscopic appearance of the glands whose weights are less than 6.0 g. reveals a broader and more prominent zona reticularis than normal, but not as broad as is found in the characteristic lesion. Moreover, cellular hypertrophy, nuclear pleomorphism or lipid infiltration of the zona reticularis is always lacking.

Small microadenomas composed more commonly of clear cells, but occasionally of compact cells, often occur not only in normal but also in hyperplastic glands. They can be situated either in the substance of the gland especially subcapsularly or in the surrounding adipose tissue of the gland. They are usually noted only on microscopical examination in contrast to the nodules of adenomatous hyperplasia which are obvious on gross inspection. The nodules of this latter entity possess a peripheral capsule derived from the gland itself, but their component cells, predominantly clear in type, are contiguous with those of the typical hyperplastic cortex found in association (Fig. 9).

Adrenal glands removed from patients with Cushing's syndrome at autopsy exhibit the characteristic changes superimposed upon which are the morphological alterations which occur in response

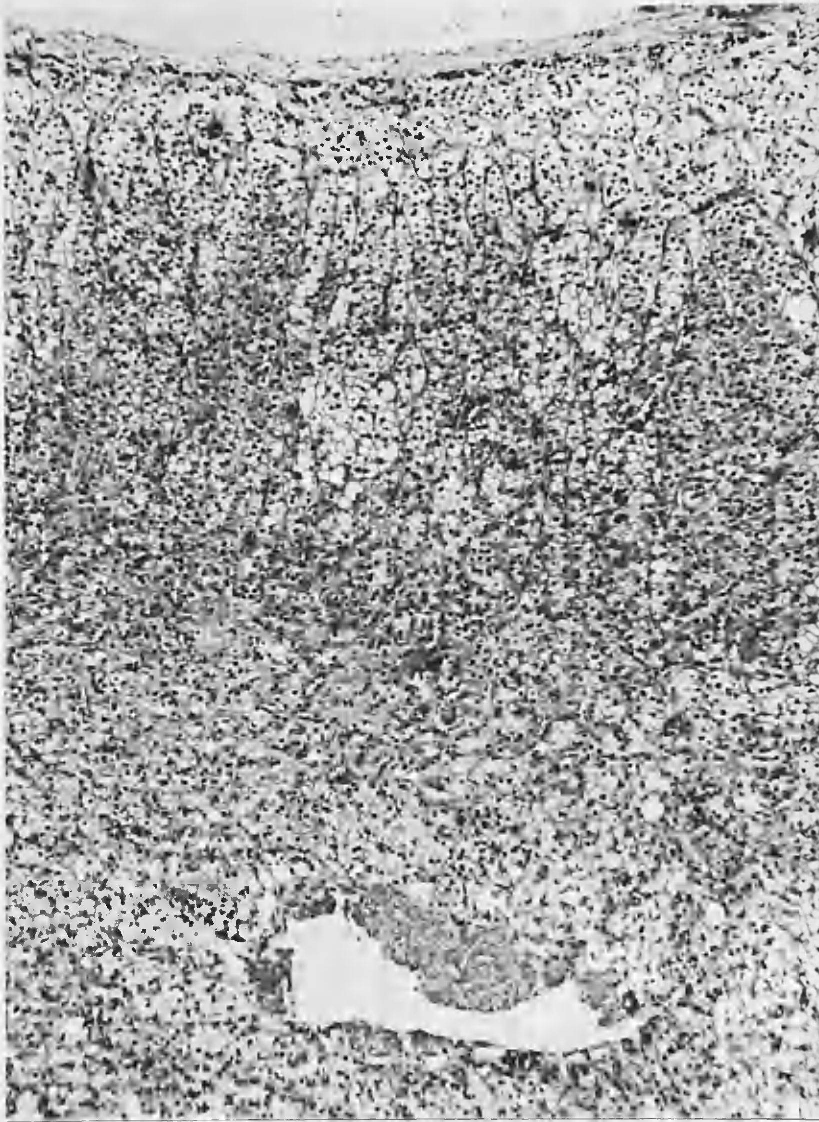


Fig. 8.- Cushing's syndrome - bilateral hyperplasia. Therapy - o,p-DDD. The inner aspect of the cortex is occupied by lipid containing cells while compact cells compose the central part. The outer aspect of the cortex consists of clear cells. H & E x100.

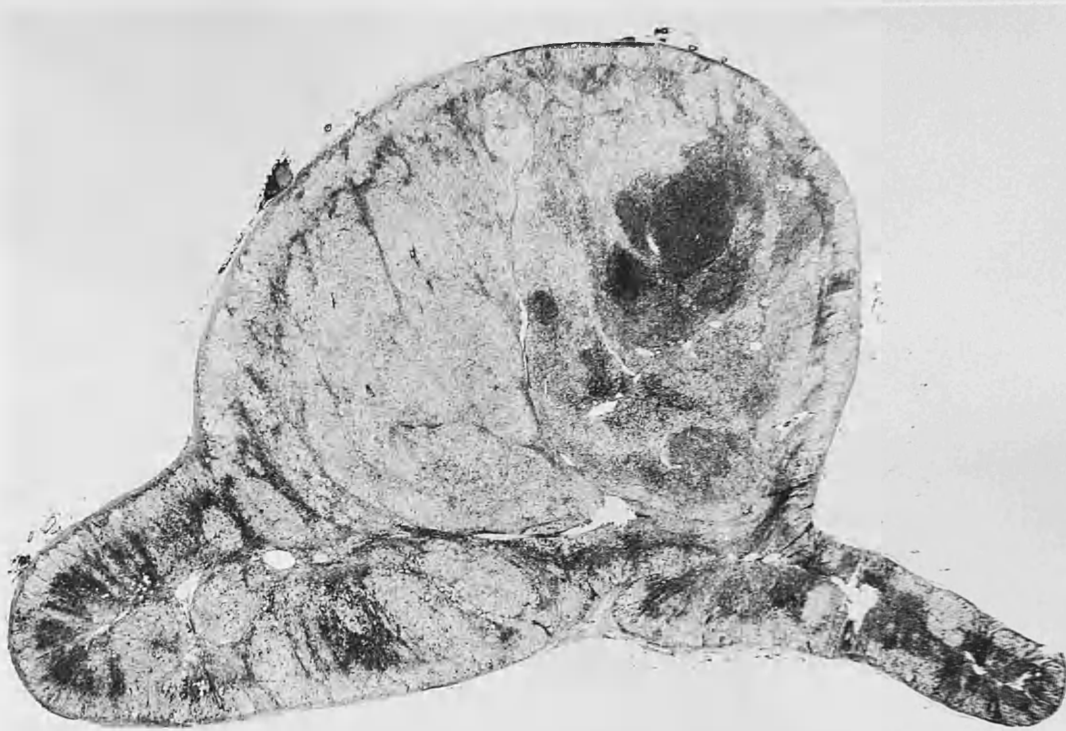


Fig. 9.- Cushing's syndrome - bilateral adenomatous hyperplasia. A prominent nodule, composed principally of clear cells but with foci of compact cells, is projecting from one aspect of the associated hyperplastic cortex. H & E x5.

to the stress of dying, so that the entire cortex consists of a unified zone of compact cells (Fig. 10).

A similar appearance, except that the cortex is usually broader, is seen in the gland removed from patients who also suffered from bronchogenic carcinoma. However, foci of clear cells often occur in the substance of the cortex or cap the columns of compact cells (Fig. 11). Both cell types frequently are hypertrophied. If metastases from the primary tumours involve the adrenal gland, they are noted first in the zona glomerulosa, the medulla or the cortical lymphatics.

The distribution of the zona glomerulosa is normal in the majority of hyperplastic glands causing Cushing's syndrome. A few, mostly those associated with pre-operative hypokalaemia, show a slight degree of hyperplasia of the zona glomerulosa.

TUMOURS OF THE ADRENAL CORTEX

The aetiology of autonomous functioning neoplasms of the adrenal cortex causing Cushing's syndrome would appear to be unrelated to the trophic hormone ACTH or the hypothalamo-pituitary axis. Such tumours may cause either a pure or a mixed type of Cushing's syndrome, in the latter virilism being an added feature. Both benign and malignant growths may cause the disease, their relative incidence being shown in Table II.

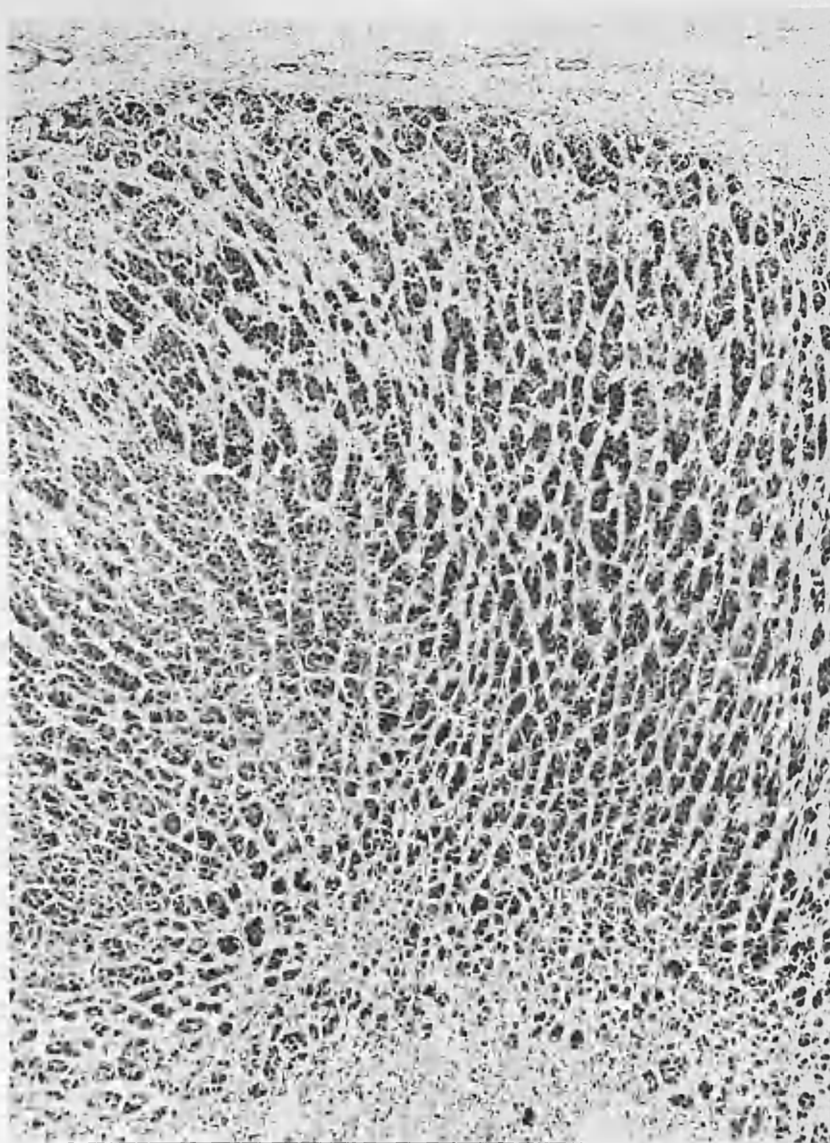


Fig. 10.- Cushing's syndrome - bilateral hyperplasia (autopsy specimen). Compact cells compose the entire cortex so that the zona reticularis and fasciculata form a unified zone. H & E x75.

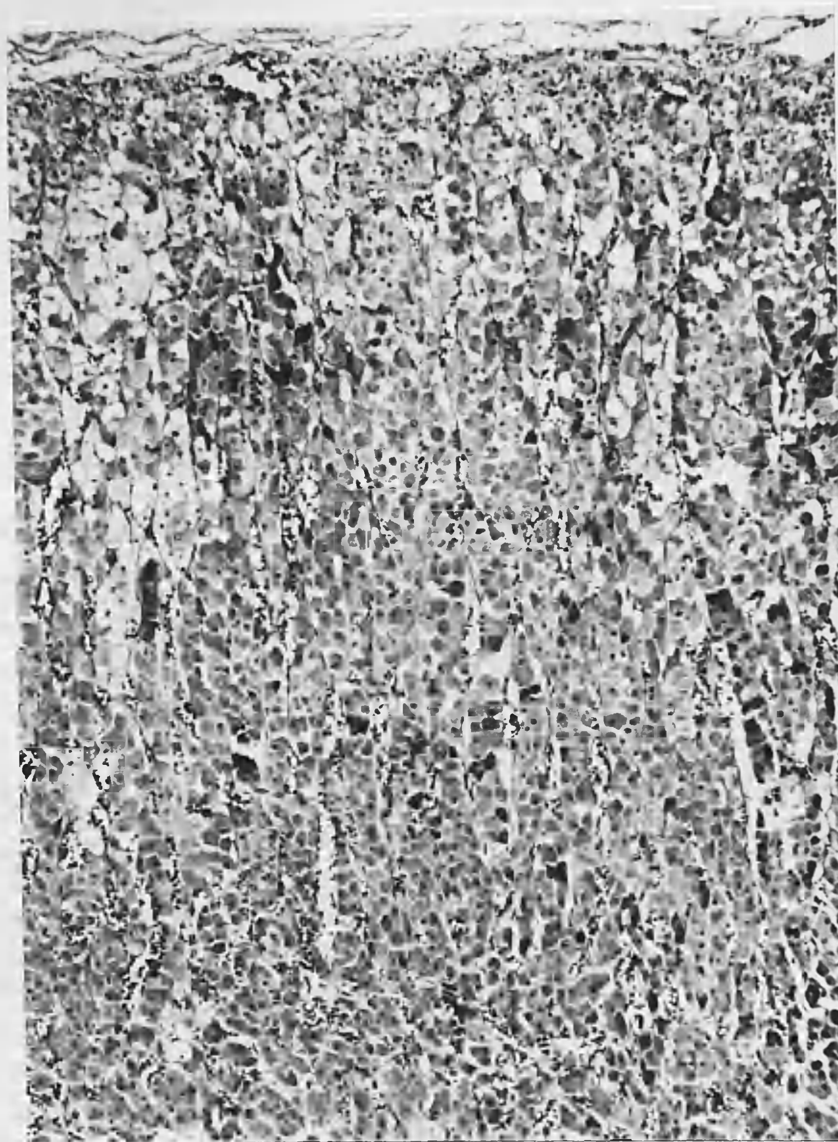


Fig. 11.- Cushing's syndrome - bilateral hyperplasia due to bronchogenic carcinoma. Compact cells, some of which are hypertrophied, others showing degenerative changes, compose the cortex, with large clear cells forming foci, either in the substance or in the peripheral parts of the cortex. H & E x100.

Benign Tumours.

Five benign adrenocortical tumours were studied in the present series (Table X). All except Case 1, were associated with the pure form of the syndrome. The preponderance of females and the age incidence of benign growths paralleled the findings in Cushing's syndrome due to bilateral hyperplasia. The clinical details of Case 5 (Table X) could not be traced. All the tumours weighed less than 40 g. and possessed well formed capsules by which they were attached to the associated thinned and atrophic adrenal cortex.

On sectioning these oval or spherical lesions, a yellow appearance is seen in which small dark brown foci are apparent. Areas of haemorrhage and necrosis are also observed, especially in Cases 2 and 5 (Table X).

Microscopic examination reveals that the predominant cell type is the clear lipid-laden cell, slightly larger in size but morphologically and histochemically similar to normal clear cells (Fig. 12). The cellular arrangement is in the form of small alveoli, cords or columns separated from one another by fibrovascular trabeculae which arise from the capsule. The small brown coloured foci noted on gross inspection consist of cells with an eosinophilic granular cytoplasm, resembling the compact cells of the zona reticularis of the normal adrenal cortex (Fig. 12).

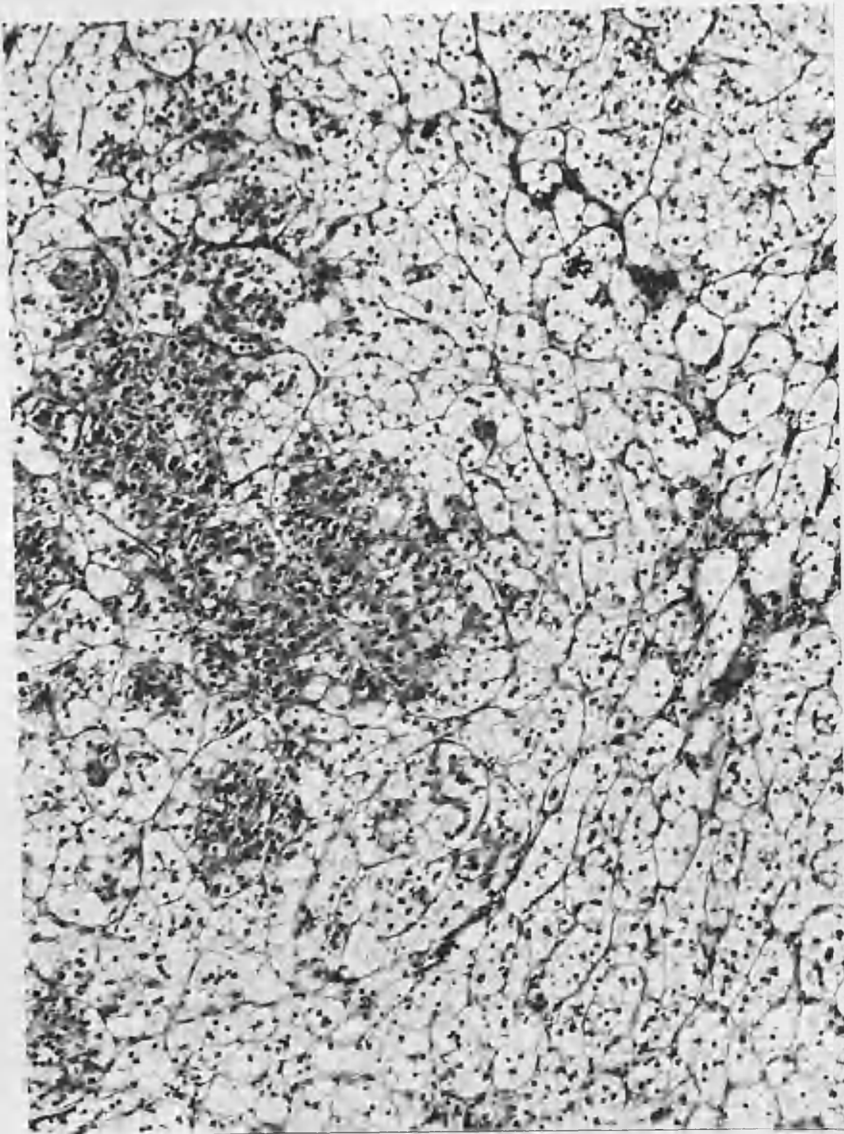


Fig. 12.- Cushing's syndrome - adenoma. The predominant component cells are clear in type with foci of compact cells. They are arranged in alveoli separated by a fibrovascular stroma. H & E x100.

These cells are arranged in similar patterns as the clear cells and with increasing size of the tumour constitute more of its structure. The nuclei of both the clear and compact cells may be normal in size but nuclear pleomorphism with bizarre types may be present. Mitoses are absent or minimal. Haemorrhage and necrosis tend to be absent from the smaller tumours, and even in large ones are infrequent.

Malignant Tumours.

Table XI demonstrates the relative data concerning six malignant tumours all of which were associated with Cushing's syndrome of the mixed type. The preponderance of females is again apparent. There is a wide age and weight distribution. The weights of the tumours in Cases 2 and 5 (Table XI) were not recorded. Both were stated to be "large". The two tumours weighing 3,000 g. and 4,040 g. measured 27.5 x 22 x 12.5 cm. and 30 x 25 x 10 cm. respectively. A well formed capsule was present in each of the tumours in which the entire specimen was available for study. The thinned remains of the adrenal gland were stretched over the tumours.

The cut surface of these growths presents a fleshy rather brown colour with irregular lobulations (Fig. 13). The tissue may be rather soft and friable especially in the larger lesions, in which areas of haemorrhage and necrosis are frequent.

TABLE XI

CUSHING'S SYNDROME

Malignant Tumours of the Adrenal Cortex

Case Number	Age Years	Sex	Site	Weight (g.)	Result
1	15	Female	Right adrenal	139	Died 3 years later with pulmonary metastases
2	19	Male	Right adrenal	- *	Died in immediate post-operative period with pulmonary, hepatic and para-aortic lymph node metastases
3	29	Female	Left adrenal	4,040	Died in immediate post-operative period with pulmonary and para-aortic lymph node metastases
4	49	Female	Right adrenal	386	Died 15 months post-operatively with pulmonary, hepatic, cerebral and lymph node metastases
5	56	Female	Right adrenal	- *	Autopsy finding: pulmonary metastasespresent
6	59	Female	Left adrenal	3,000	Alive 6 months after operation

*Details not available

Fig. 13.- Cushing's syndrome - carcinoma (weight 139 g.). The tumour is encapsulated and divided into irregularly shaped lobules. It is soft and fleshy and shows areas of necrosis.

The carcinomas are composed of cells of the compact type, little lipid being demonstrable in their eosinophilic cytoplasm. While neither acid nor alkaline phosphatase can be stained in these cells, the Δ_5-3β-hydroxysteroid dehydrogenase is present in trace amounts as judged histochemically. RNA is apparently absent and mitochondria are sparse. The cells are arranged in alveoli of varying size and shape (Fig. 14) although cords and columns of cells may be seen in some areas. A consistent feature in each tumour is the presence of enlarged vesicular ovoid or round nuclei which possess a finely stippled interior in which one or more nucleoli are present (Fig. 15). Although uniformity of cell and nuclear size and shape can be observed in some areas, most zones exhibit cellular and nuclear pleomorphism. Giant forms occur, but mitotic figures are not prominent in any of the lesions. Areas of necrosis are obvious in each of the malignant tumours especially the tumours of Cases 4 and 6 (Table XI). The necrosis involves large numbers of cells in contrast to the individual cell necrosis seen in growths which subsequently behave in a benign manner (Fig. 16). Haemorrhage, both recent and old, the latter containing cholesterol clefts and haemosiderin laden macrophages, is seen in several of the tumours.

Each tumour possesses a well formed capsule from which fibrous trabeculae carrying blood vessels arise to divide the

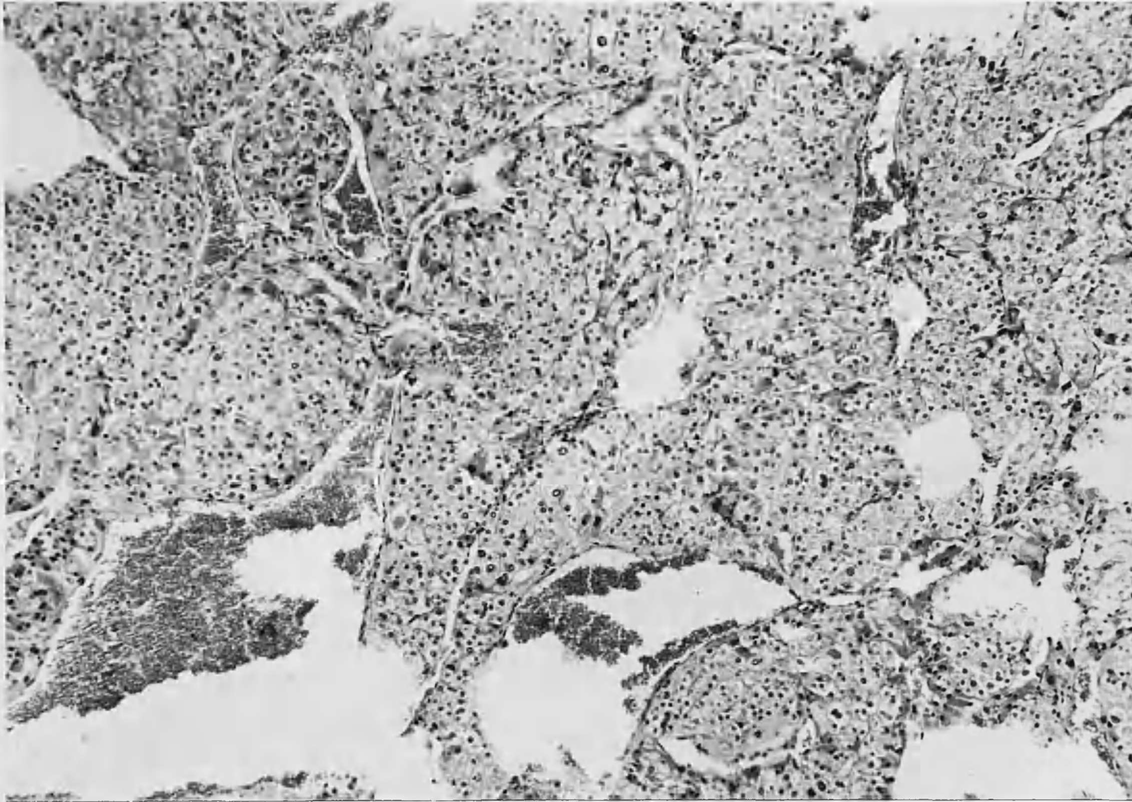


Fig. 14.- Cushing's syndrome - carcinoma. The tumour cells are arranged in alveoli of varying size, separated by trabeculae in which dilated vascular spaces are present. H & E x95.

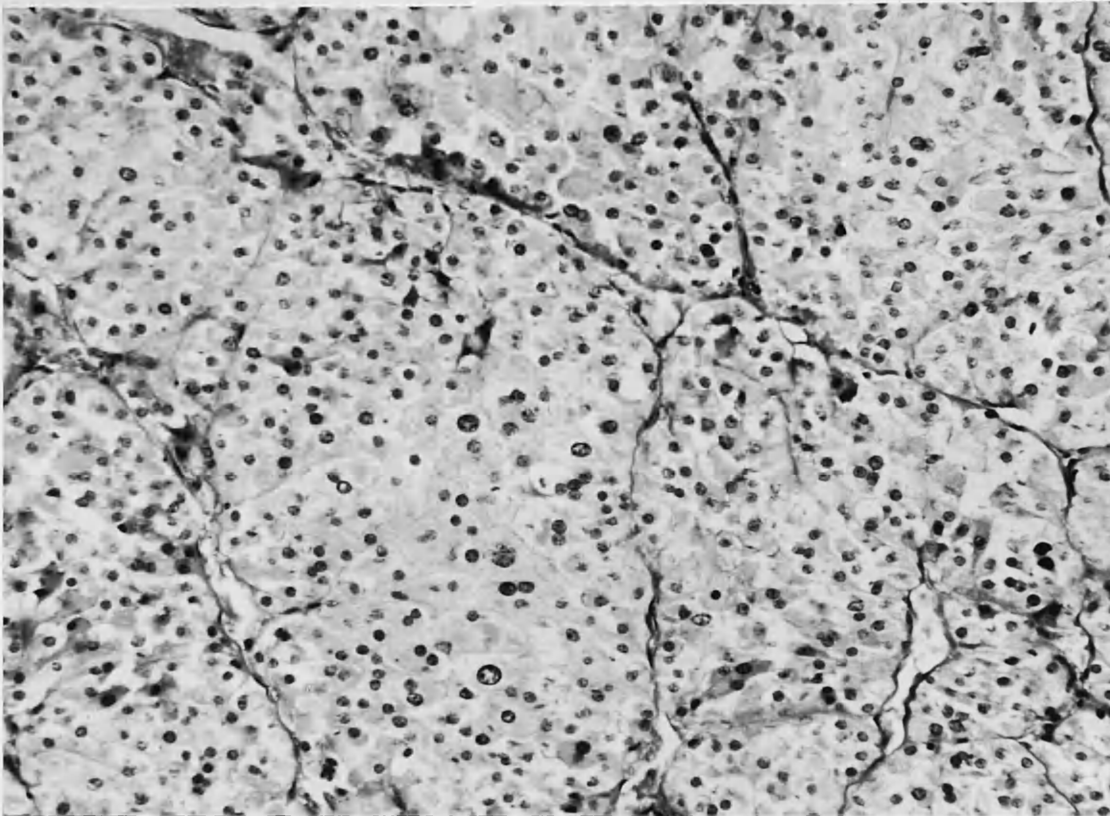


Fig. 15.- Cushing's syndrome - carcinoma. The alveolar arrangement of the cells is more obvious. The tumour cells are compact in type, but are larger than normal compact cells. Their nuclei are vesicular in character and exhibit pleomorphism. H & E x240.

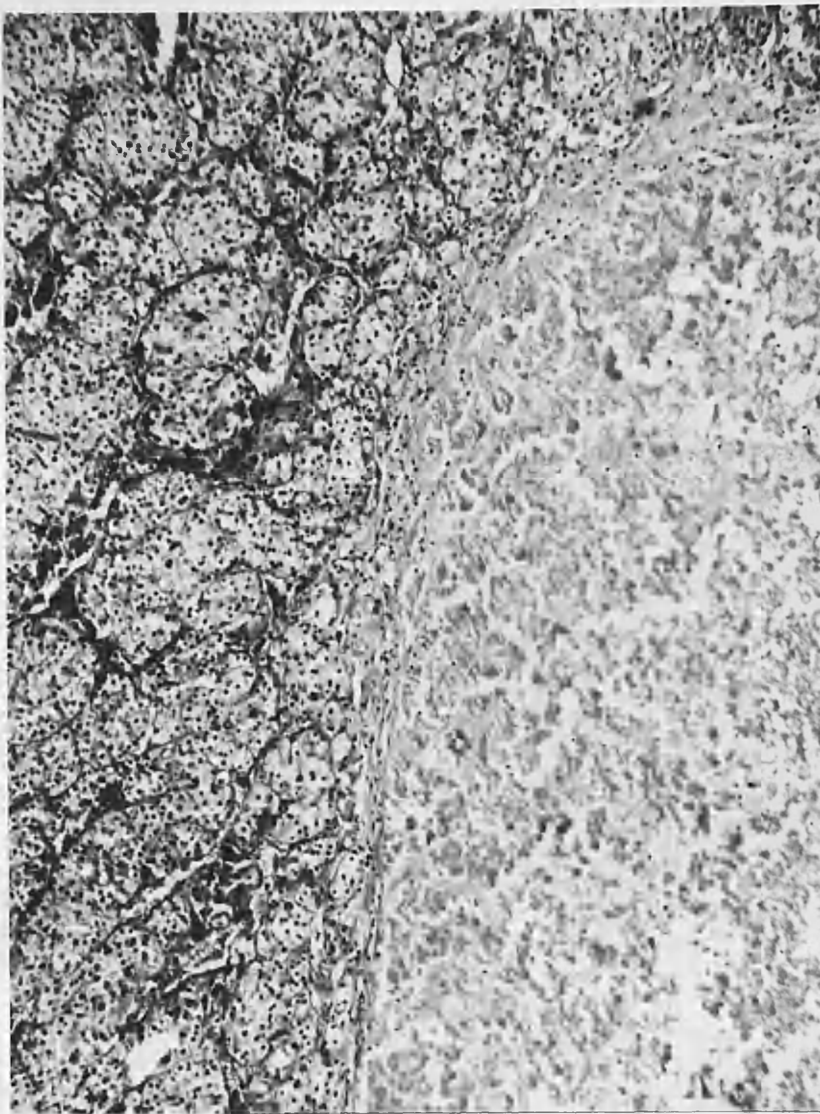


Fig. 16.- Cushing's syndrome - carcinoma. The alveolar arrangement of the compact cells is seen together with an extensive area of necrosis. H & E x100.

tumours into lobules. Many dilated vascular channels are present in these tumours (Fig. 14) but in none is there any evidence of vascular invasion by the malignant cells.

Local invasive infiltration occurred in each of the lesions examined at autopsy. In one patient (Case 4, Table XI) the tumour extended from the bed of the right adrenal gland through the diaphragm to the pleura. Lymph node metastases occurred in three patients (Cases 2-4, Table XI) and in five, pulmonary metastases were noted. The liver and brain were involved in two cases (Cases 2 & 4, Table XI) (Fig. 17). Case 6 is included as a malignant tumour in the absence of proved metastases on the strength of extensive necrosis and large prominent vesicular nuclei. These two features are the only consistent histological guides to malignancy shared by the five cases of carcinoma in which malignancy was proved by the capacity to metastasise.

The Associated Adrenal Cortex.

Evidence of cortical atrophy is observed in the associated adrenal cortex found in conjunction with functioning benign and malignant tumours. The capsule of the gland is broad and oedematous and the cortex consists of a narrow zone of clear cells only (Fig. 18).

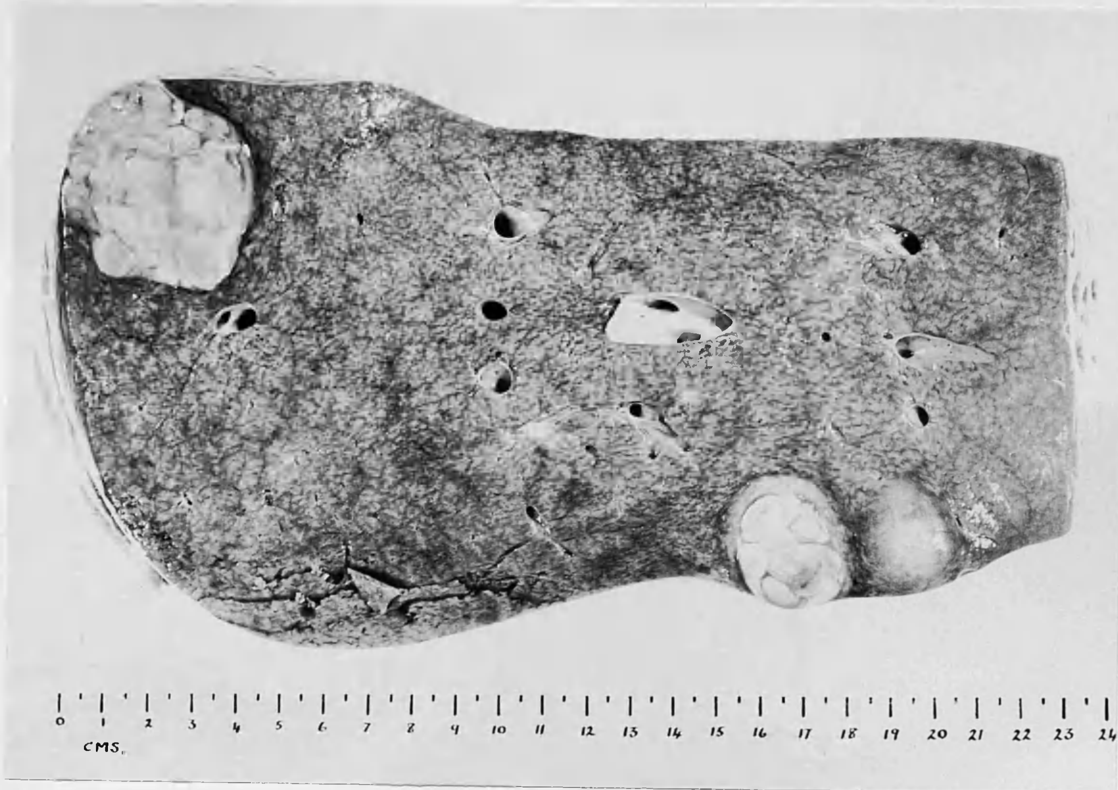


Fig. 17.- Cushing's syndrome - carcinoma. Metastases involving the liver.

Fig. 18.- Cushing's syndrome - adrenal gland found in association with benign or malignant tumours. The associated adrenal gland shows evidence of steroid induced atrophy, with the cortex composed solely of clear cells. The capsule of the gland is thickened and oedematous. H & E x65.

THE PATHOLOGY OF THE ADRENOGENITAL SYNDROME

A. VIRILISM

The adrenogenital syndrome accounted for 17 of the total of 122 cases of hypercorticalism (Table I). Ten of these patients had benign or malignant tumours of the adrenal cortex, while seven were due to bilateral hyperplasia. The relative incidence in this series gives a false indication of incidence of the two pathologies in general. Between 1943 and 1962, 435 cases of congenital adrenal hyperplasia were reported (Bratand & Thompson, 1943; Bentwick et al., 1952; Russell, 1954; Wilkins, 1962), whereas over a 25 year period, only 113 adrenal tumours causing the adrenogenital syndrome were found in a review of the literature (Rapaport et al., 1952; Heinbecker et al., 1957). Doubtless, due to the simplicity of effective therapy for congenital adrenal hyperplasia, many more cases remain unrecorded. This pathological entity is due to a congenital anomaly inherited as an autosomal recessive characteristic which manifests itself as a deficiency of one of the enzyme systems involved in the biosynthesis of cortisol.

BILATERAL ADRENOCORTICAL HYPERPLASIA

All seven cases of congenital adrenal hyperplasia in the present series occurred below the age of thirteen years, and all

but one were females, two of whom were sisters. The remaining patients were unrelated.

The weights were recorded only in the glands removed from two patients. In one, a female aged twelve years, they weighed 21.8 g. and 20.0 g. In the other patient, only one gland was examined and it was found to weigh 7.5 g. This small increment in weight was due to the patient having received therapeutic suppressive doses of cortisone pre-operatively.

Macroscopically, the adrenal cortex is lobulated and the surface is thrown into a series of convolutions. The edges are rounded and the cut surface is uniformly brown in colour, resembling the gross appearance of the zona reticularis of the normal gland.

Histological examination of these glands reveals the presence of a diffusely broadened cortex composed exclusively or almost exclusively, of compact cells. Those cells exhibit the same histochemical enzymic properties as the compact cells of the normal gland and similarly have abundant mitochondria and stainable RNA. This cellular pattern extends from the inner border of the cortex to the zona glomerulosa (Fig. 19). In a few focal area the columns of compact cells are capped by a few clear cells. The zona glomerulosa is prominent and hyperplastic in all but one of the glands of this series. It is present around the entire

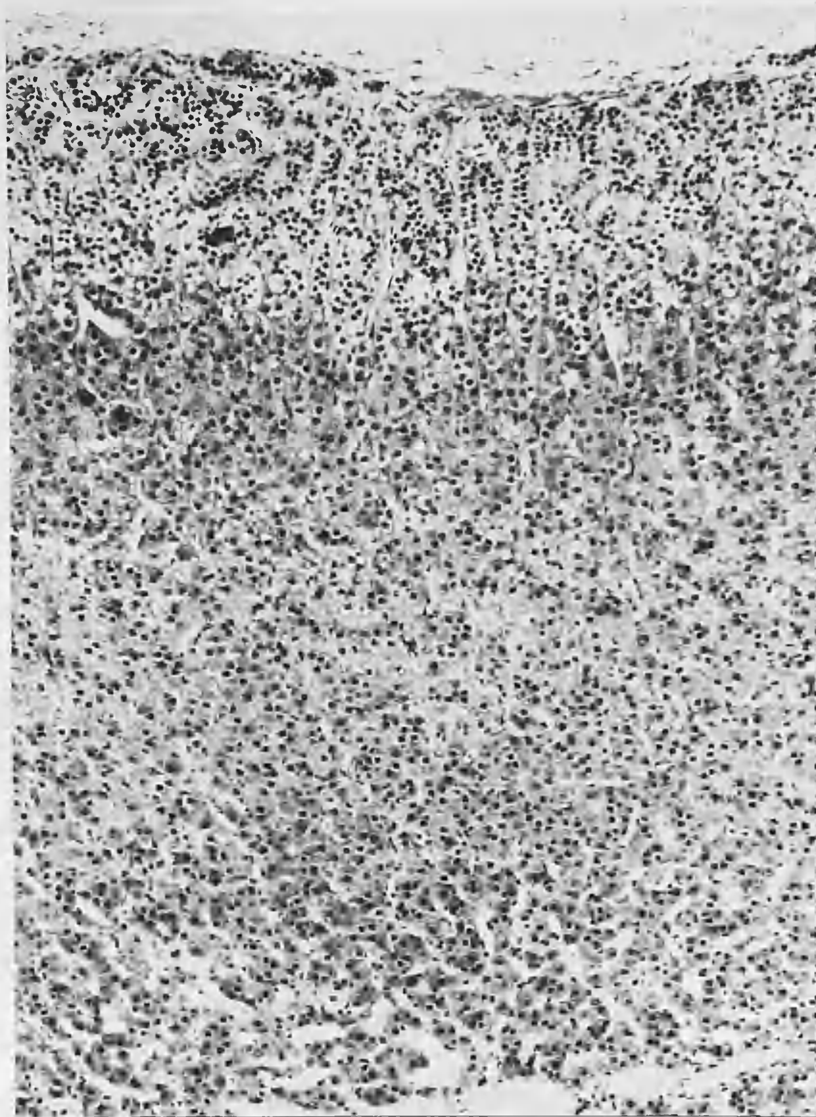


Fig. 19.- Adrenogenital syndrome - bilateral hyperplasia. A prominent, hyperplastic zona glomerulosa is present. Compact cells compose the remainder of the cortex and form a unified zone. H & E x150.

periphery of the cortex and is between two and four times its normal width (Fig. 19).

TUMOURS OF THE ADRENAL CORTEX

The adrenogenital syndrome when caused by adrenocortical tumours occurs particularly before the age of twelve years and more frequently in females than males (Goldstein *et al*., 1946; Rapaport *et al*., 1952; Heinbecker *et al*., 1957; Symington & Jeffries, 1962). Both benign and malignant tumours may cause the syndrome and from the review series published by Rapaport (1952) and Heinbecker (1957) and their colleagues, malignant lesions appear to occur at least twice as frequently as benign ones.

Benign Tumours.

Adrenocortical adenomas were responsible for six cases of the syndrome in this series (Table XII). Although the sex distribution was equal, four of the tumours were found in patients less than twelve years of age. The range of recorded weights of the growths showed a wide scatter and in the larger tumours found in Cases 3 and 4 (Table XII), difficulty was encountered in deciding whether they were benign or malignant.

All the adenomas are surrounded by a well formed capsule to which the associated atrophic adrenal cortex is attached.

TABLE XII

THE ADRENOGENITAL SYNDROME

Benign Virilising Tumours of the Adrenal Cortex

Case Number	Age Years	Sex	Site	Weight g.	Result
1	5	Female	Right adrenal	25	Well 9 years later
2	8	Male	Left adrenal	80	Well 3 months later
3	8	Female	Right adrenal	400	Well 1½ years later
4	8	Male	Left adrenal	228	Well 2½ years later
5	15	Male	Left adrenal	47	Well 4 months later
6	29	Female	Right adrenal	35	Well 8 years later

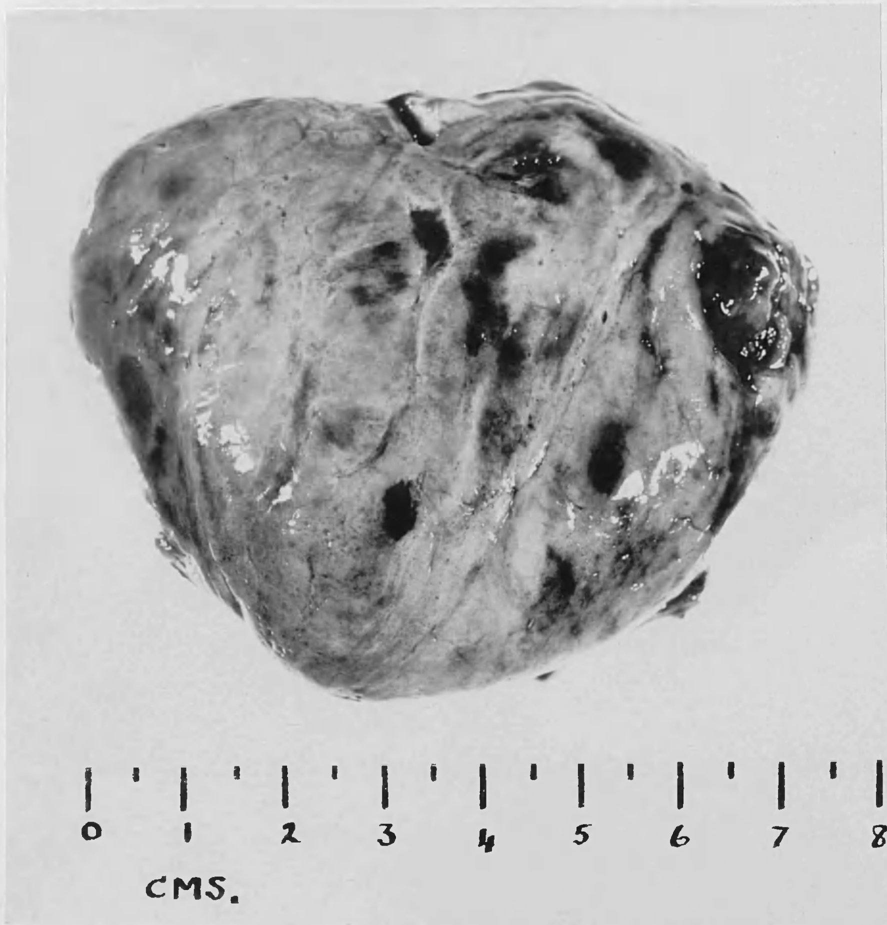


Fig. 20.- Adrenogenital syndrome - adenoma (228 g.). The tumour is encapsulated. It is soft in consistency and shows areas of recent haemorrhage.

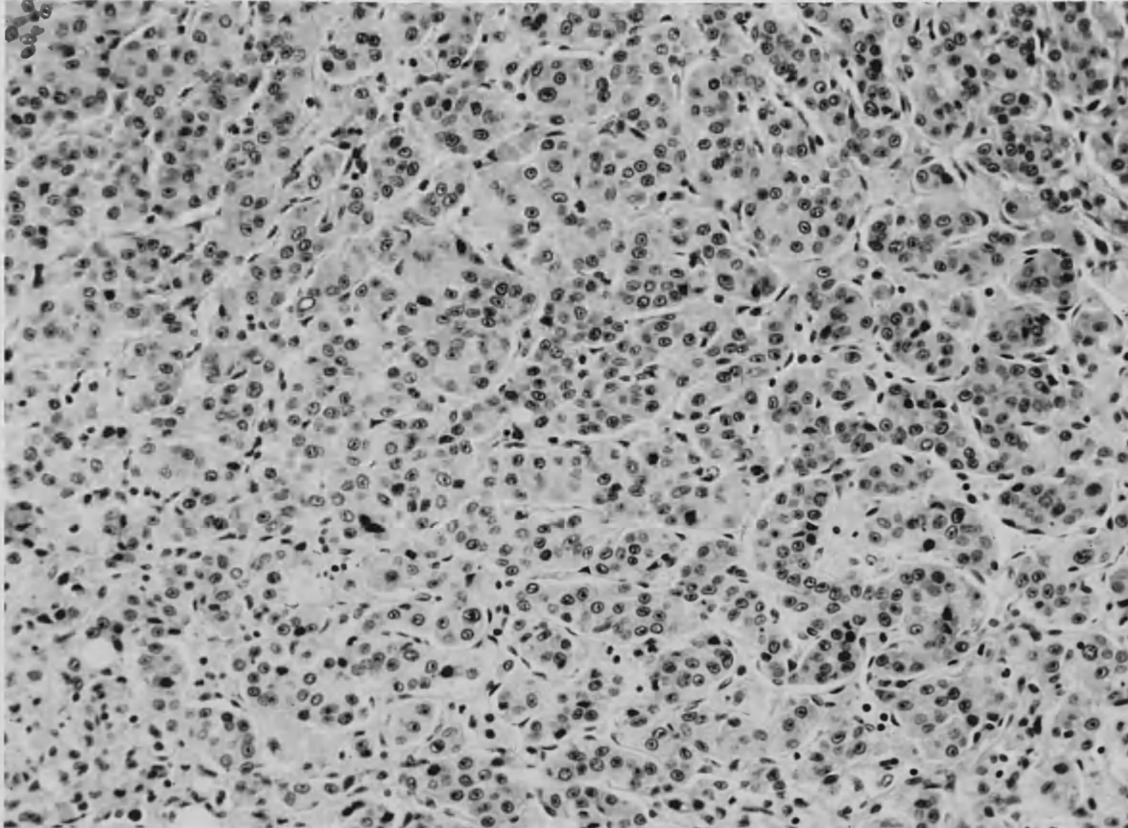


Fig. 21.- Adrenogenital syndrome - adenoma. The cells are compact in type and are arranged in small alveoli or cords. Little nuclear or cellular pleomorphism is present. H & E x220.

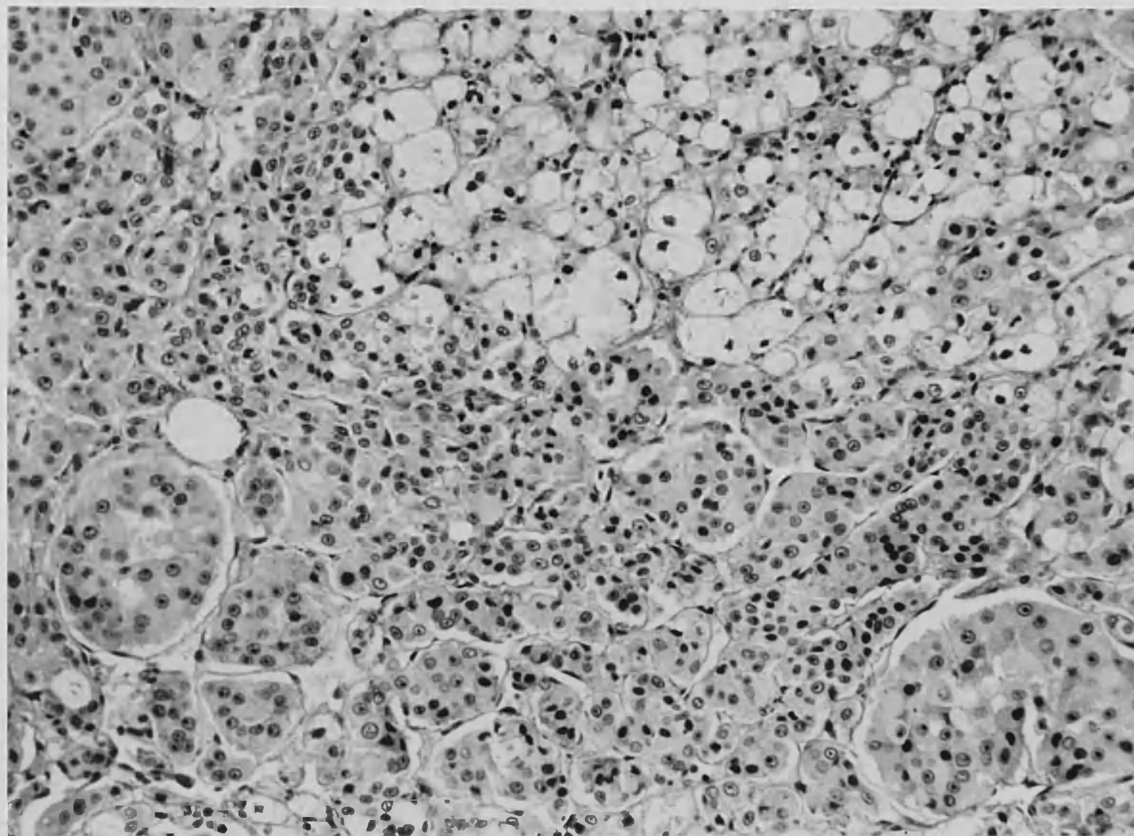


Fig. 22.- Adrenogenital syndrome - adenoma. Both clear and compact cells are present in this lesion, the latter predominating. They are arranged in alveoli or cords. Some nuclear pleomorphism is seen. H & E x225.

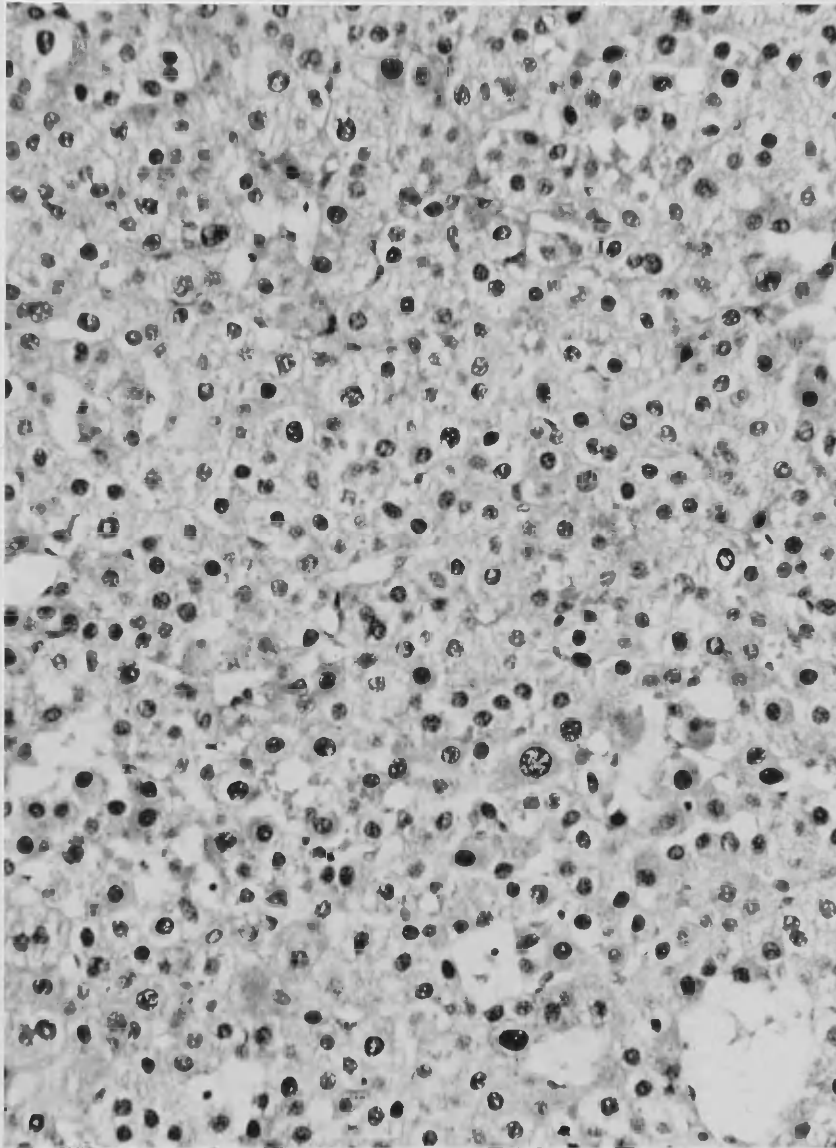


Fig. 23.- Adrenogenital syndrome - adenoma (228 g.). The cells are compact in type and are larger than normal. Nuclear pleomorphism is prominent and many of the nuclei are enlarged and vesicular in character. Dense pyknotic nuclei are present in some cells, the cytoplasm of which is eosinophilic and homogeneous. H & E x240.

absent or minimal in all the tumours, and necrosis which is confined to individual cells in the smaller growths tends to be more extensive.in the larger tumours of Cases 3 and 4 (Table XII). Fibrovascular trabeculae, which are derived from the well formed capsules, divide the tumour into lobules and surround each of the alveoli (Fig. 21).

It is not clear whether the tumours from Cases 3 and 4 (Table XII) are benign or malignant. The histological picture is complicated by the presence in both tumours of large areas of necrosis and the presence of larger vesicular nuclei in Case 4 (Table XII). This child shows no evidence of recurrence 30 months after the removal of the tumour, but the outcome must be awaited before a final decision can be reached. In Case 3 (Table XII), although necrosis is prominent in the tumour, the nuclei are small and not enlarged so that the diagnosis of a benign growth can be made with more certainty.

Malignant Tumours.

All the patients in whom carcinomas of the adrenal gland were responsible for the development of the adrenogenital syndrome were females (Table XIII). Benign and malignant tumours associated with this syndrome shows a considerable overlap,

TABLE XIII

THE ADRENOGENITAL SYNDROME

Malignant Virilising Tumours of the Adrenal Cortex

Case Number	Age Years	Sex	Site	Weight g.	Result
1	3	Female	Left adrenal	265	Died 3 years post-operatively with large functional mass at the site of original tumour. No autopsy.
2	12	Female	Right adrenal	500	Died 4 months post-operatively with hepatic and cardiac metastases
3	25	Female	Right adrenal	1,500	Died in immediate post-operative period. No metastases apart from bilateral local involvement.
4	58	Female	Left adrenal	1,250	Autopsy finding, pulmonary and hepatic metastases present.

in weight (Tables XII & XIII). Two of the lesions (Cases 2 & 4, Table XIII) were proved malignant by the presence of metastases. Apart from local involvement of the tumour site and the presence of a morphologically similar tumour in the opposite adrenal gland, no metastases were found at the autopsy of Case 3 (Table XIII). No clinical evidence of secondary tumour was noted in Case 1 (TABLE XIII) apart from its recurrence at the original site.

The carcinomas are all well encapsulated and on sectioning, the cut surface presents a fleshy appearance in which areas of necrosis and haemorrhage are prominent particularly in the larger lesions (Fig. 24). The associated adrenal glands are noted in relation to one pole of the tumour and are stretched over their surface.

The cell component common to all of those tumours is the compact type of cell in which lipid is absent from or sparse in the cytoplasm. The cells are larger than the compact cells of the zona reticularis of the normal gland and marked pleomorphism is present with the formation of giant cells in some areas (Fig. 25). The nuclei are also enlarged and their vesicular character is a prominent feature (Fig. 26). One or more nucleoli are noted in the nuclei which also have a well marked nuclear membrane. Mitoses are not frequent in any of the lesions. In some areas, particularl in the tumour removed from Case 1 (Table XIII), a remarkably

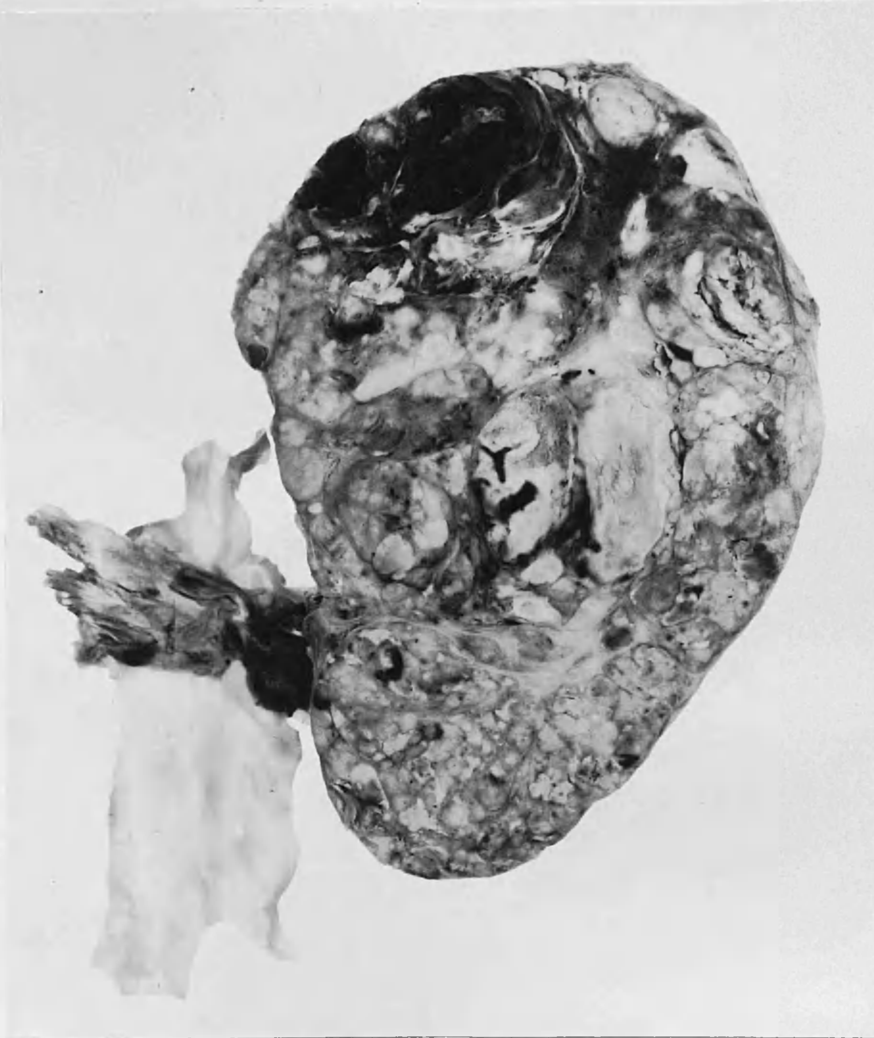


Fig. 24.- Adrenogenital syndrome - carcinoma (1,250 g.)
The tumour is encapsulated and divided into lobules.
Areas of haemorrhage and necrosis are prominent.

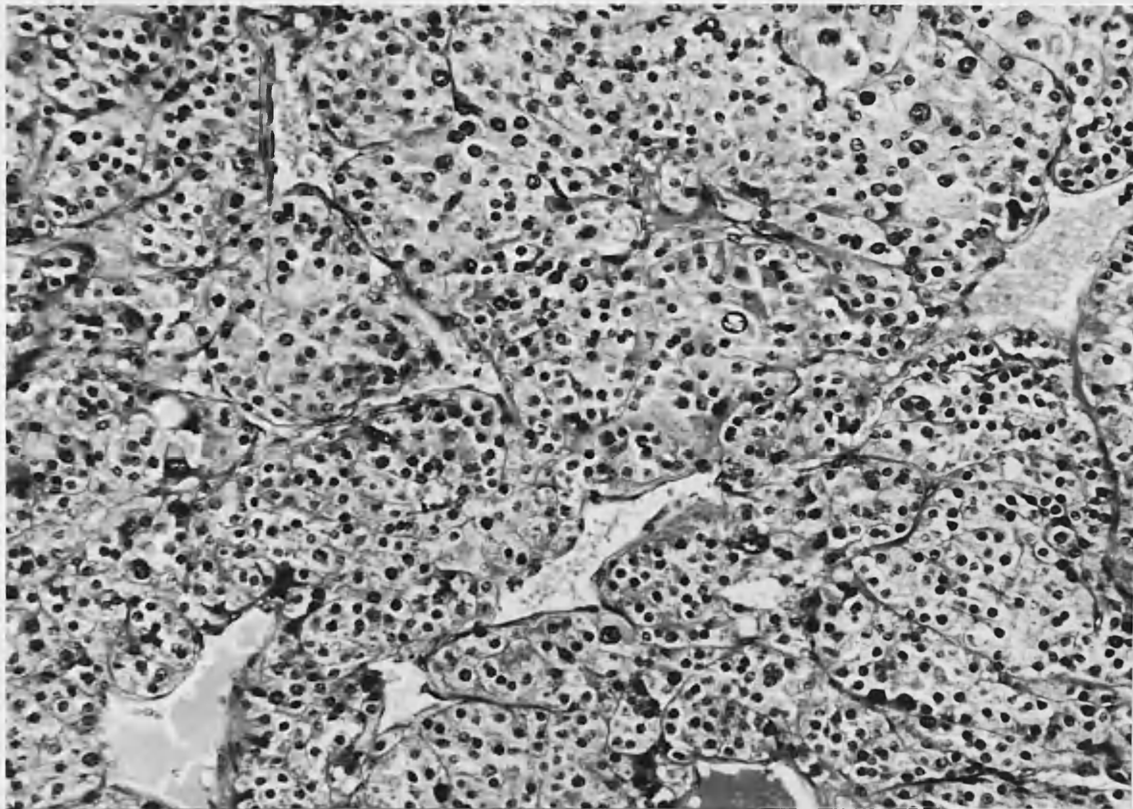


Fig. 25.- Adrenogenital syndrome - carcinoma. The compact cells are larger than normal and are arranged in alveoli separated by fibrovascular trabeculae. Nuclear pleomorphism is marked and giant forms are present. H & E x240.

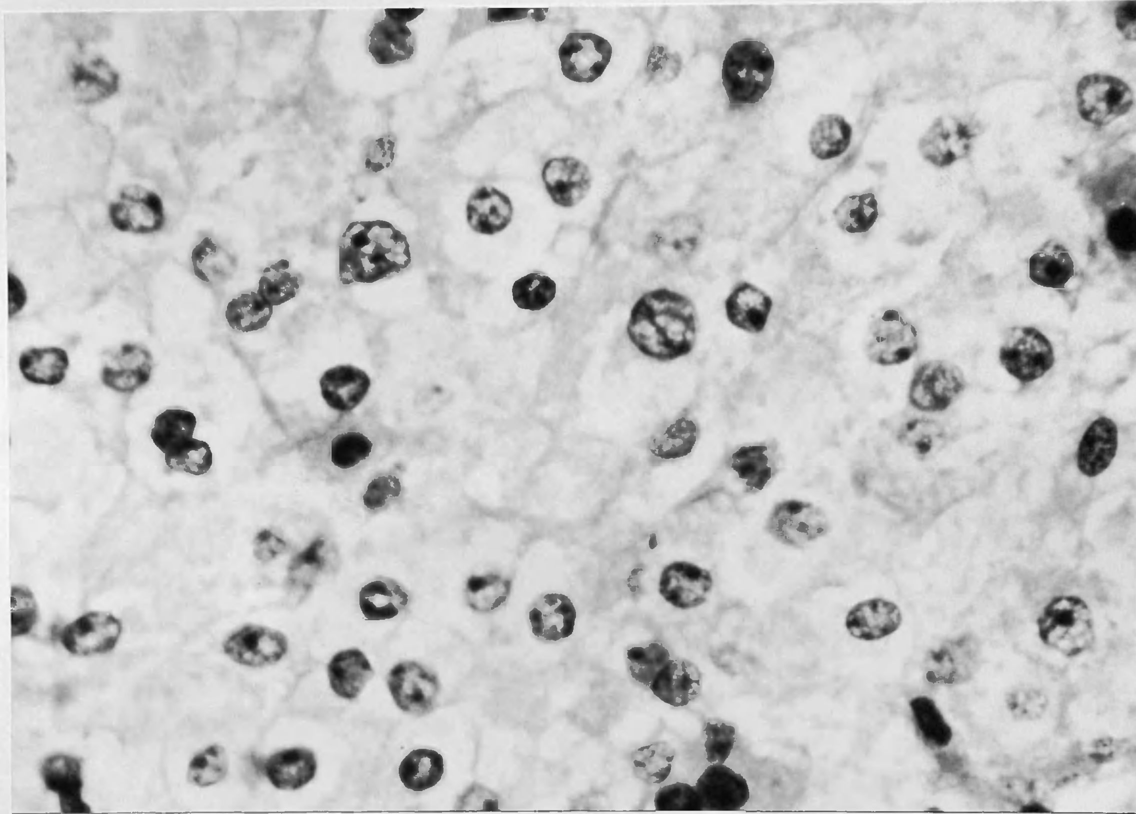


Fig. 26.- Adrenogenital syndrome - carcinoma. The component compact cells possess prominent vesicular nuclei, in which one or more nucleoli may be seen. Pleomorphism is also present. H & E x950.

uniform nuclear and cellular pattern can be observed in which the cells are very similar to the normal compact cell and in which the nuclei are round and small. Histochemical demonstration of the Δ_5-3β-hydroxysteroid dehydrogenase enzyme in the tumour of Case 3 (Table XIII) yields negative results. The cellular arrangement differs not only between the tumours but also in any one tumour. The formation of alveoli can be less conspicuous than in the benign tumours of this syndrome, and cords or syncitial groups of cells may be more apparent. Extensive areas of necrosis involving many groups of cells are apparent and haemorrhagic zones are also seen. Dilated vascular sinusoidal spaces are prominent and lie in close apposition to the tumour cells. In none of these malignant growths is there evidence of vascular invasion. Although the tumours have a lobular structure, fibrovascular trabeculae are not prominent among the syncitial cell areas, but can be seen surrounding the alveolar structures.

The Associated Adrenal Cortex.

The appearance of the associated adrenal cortex is similar to that occurring in association with Cushing's syndrome due to autonomous functioning tumours (Fig. 18), except that a prominent hyperplastic zona glomerulosa is observed in the atrophic cortex attached to the tumour removed from Case 4 (Table XII).

B. FEMINIZATION

Feminizing tumours of the adrenal cortex are the least common manifestation of the adrenogenital syndrome, a total of 52 cases having been recorded and reviewed by Gabrilove and his colleagues (1965). Of these, 10 appeared to be benign, while of the remainder, 30 were proved to be malignant by the presence of metastases at autopsy.

TUMOURS OF THE ADRENAL CORTEX

Two male patients of the present series of 122 examples of hypercorticalism presented with symptoms and signs of feminization. Both were due to tumours, one of which has been found to be malignant (Table XIV).

Both tumours are encapsulated and divided into lobules of differing size and shape. Areas of haemorrhage and necrosis are conspicuous. The tissue is soft and is brown to pink in colour.

A remarkable similarity exists between the two tumours in cell type and structure. The neoplastic cells are compact but are larger than normal. They are arranged in sheets or cords with little intervening fibrovascular stroma. The nuclei are large and vesicular with prominent nucleoli and nuclear membranes (Fig. 27) Mitoses are rare. A minimal degree of pleomorphism is present

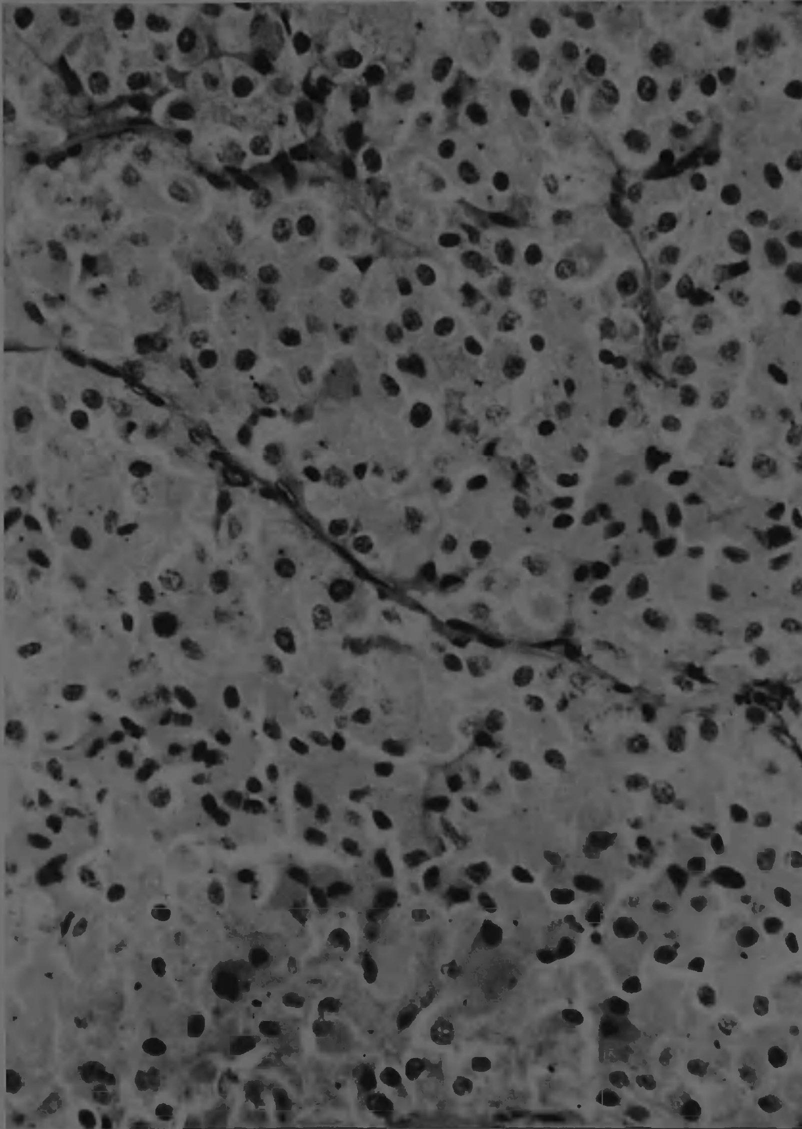


Fig. 27.- Feminization - carcinoma. The cells are compact in type and have enlarged vesicular nuclei. Little cellular or nuclear pleomorphism is present. Many cells possess pyknotic nuclei and an homogeneous eosinophilic cytoplasm. H & E x390.

in both lesions. Although prominent areas of necrosis are seen, individual cells in many foci also show degenerative changes and necrosis (Fig. 27).

Due to the similarity of the two tumours, particularly with regard to the enlarged vesicular nuclei and the prominent areas of necrosis, the tumour from Case 2 (Table XIV) is regarded as a malignant growth despite both the lack of metastases at present and the lack of pleomorphism and mitotic activity.

THE PATHOLOGY OF CONN'S SYNDROME

Conn's syndrome accounted for 23 of the total of 122 cases of hypercorticalism, of which 19 were due to adrenocortical tumours. Two of the tumours were malignant.

Since the first description of this disease in 1955 (Conn, 1955; Foye & Feichtmeir, 1955), its recognition has increased sharply. In 1964, Conn and his colleagues reviewed 145 cases due to adrenal tumours reported in the literature and they were aware of the existence of at least 70 other unreported similar lesions. Consequently, at present, it is still too early to assess the true incidence of aldosterone secreting tumours in patients with hypertension. The syndrome can also be caused by bilateral adrenocortical hyperplasia. This entity occurs more commonly in young males who present with malignant hypertension (Conn & Conn, 1961).

BILATERAL ADRENOCORTICAL HYPERPLASIA

The existence of bilateral hyperplasia of the zona glomerulosa was suspected in 4 patients in the present series. Two of the cases occurred in young male children in whom bilateral adrenalectomy produced clinical improvement. Only one gland was removed from each of the remaining two cases, one a male, the other a female, both aged 40 years. Their post-operative courses

are being studied at present as the adrenalectomies were performed on an empirical rather than scientific basis.

All the glands are normal in appearance and weight. In the two children, the zona glomerulosa is hyperplastic. It is present around the entire periphery of the cortex forming a broad zone several cells in depth (Fig. 28) from which occasional tongue-like projections may be sent into the zona fasciculata. Focal hyperplasia of the zona glomerulosa is seen in the gland removed from the 40 year old male subject, while a relatively narrow zona glomerulosa with focal areas of hyperplasia is present around the periphery of the gland removed from the fourth case.

The cells of the zona glomerulosa in cases of Conn's syndrome due to bilateral hyperplasia are identical with those of the normal gland. They are small in size, possess dense hyperchromatic nuclei and finely granular eosinophilic cytoplasm in which lipid is demonstrable. Neither mitoses nor pleomorphism are apparent. The presence of the Δ_5-3β-hydroxysteroid dehydrogenase can be noted histochemically.

The zona fasciculata and zona reticularis are normal in appearance in these glands (Fig. 28).

TUMOURS OF THE ADRENAL CORTEX

A detailed analysis of the 19 patients in whom tumours of the adrenal cortex were responsible for their developing Conn's syndrome

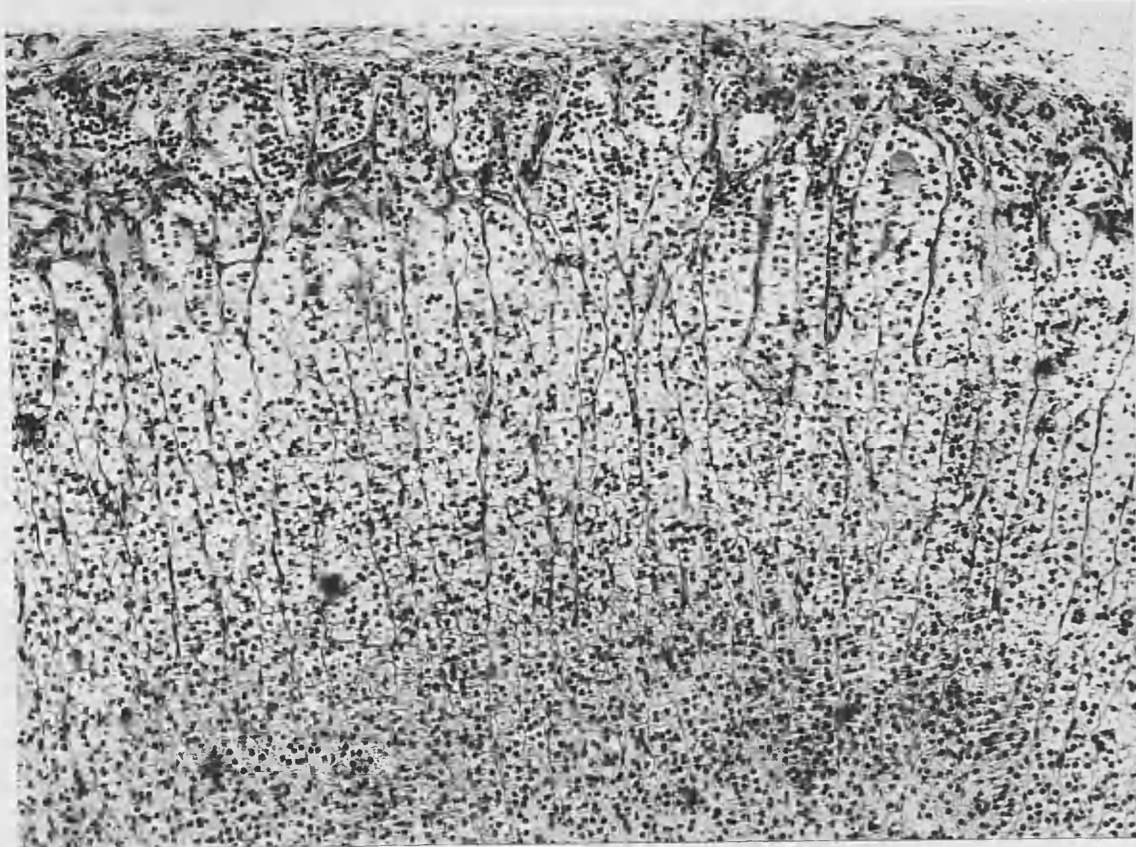


Fig. 28.- Conn's syndrome - bilateral hyperplasia. The zona glomerulosa forms a broad hyperplastic zone immediately below the capsule of the gland. The zona reticularis and fasciculata are normal in appearance. H & E x140.

is presented in Table XV. Cases 1 to 17 were caused by benign tumours while the remaining two patients had malignant growths, that of Case 18 being proven by the development of metastases post-operatively. This case has been the subject of a previous report (Brooks et al., 1957) and only 4 other malignant tumours have been recorded in the literature (Foye & Feichtmeir, 1955; Zimmerman et al., 1959; Conn et al., 1964; Leutscher, 1964).

The preponderance of females in this series closely corresponds with the incidence in general. Fourteen of the present cases occurred in women and from the published reports of Conn (1963) and Leutscher (1964), 126 of the cases were found in the female sex. The highest age incidence was between 31 and 50 years into which group fell 15 of the 18 patients in whom the weight of the tumour is known. In Table XVI, the present series is summated with 157 other patients and of the total of 175, 132 cases were found to occur in this age group. This age incidence applied equally to men and women. A predilection for affecting the left adrenal gland was noted in women not only of the present series but also from the reviewed material (Table XVII). In males, on the other hand, the tumours occurred equally on either side when single tumours were studied but in cases where multiple growths were observed, the right gland was more often involved (Table XVIII).

Whereas tumours causing the adrenogenital or Cushing's syndrome

TABLE XV

CONN'S SYNDROME

Analysis of 19 Tumours of the Adrenal Cortex

Case Number	Age Years	Sex	Site	Weight (g.)	Benign or Malignant Tumours
1	37	Female	Left adrenal	30.0	Benign
2	45	Male	Right adrenal	2.50	Benign
3	45	Female	Right adrenal	(2 cm. diam.)	Benign
4	*	Female	*	*	Benign
5	35	Female	Left adrenal	1.95	Benign
6	54	Female	*	*	Benign
7	46	Female	Left adrenal	0.80	Benign
8	45	Female	Left adrenal	3.40	Benign
9	48	Female	Left adrenal	4.80	Benign
10	40	Female	Left adrenal	30.0	Benign
11	40	Male	Right adrenal	79.0	Benign
12	40	Female	Left adrenal	(1.5 cm. diam.)	Benign
13	39	Female	Right adrenal	8.70	Benign
14	46	Female	Right adrenal	11.85	Benign
15	59	Male	Right adrenal	9.80	Benign
16	41	Female	Left adrenal	8.0	Benign
17	33	Male	Left adrenal	5.0	Benign
18	26	Female	Right adrenal	2032.0	Malignant
19	35	Male	Left adrenal	1400.0	Malignant

* Details not available

TABLE XVI

CONN'S SYNDROME

175 Tumours of the Adrenal Cortex: Age Distribution

FEMALES

Source	Age (years)							
	15-20	21-30	31-40	41-50	51-60	61-70	71-80	Total
Review publication Conn, 1963	4	9	32	42	6	4	1	98
Conn, 1963	1	1	4	5	-	-	-	11
Luetscher, 1964	-	-	3	1	-	-	-	4
Present series	-	1	5	6	1	-	-	13
Total	5	11	44	54	7	4	1	126

MALES

Source	Age (years)							
	15-20	21-30	31-40	41-50	51-60	61-70	71-80	Total
Review publication Conn, 1963	-	6	10	14	4	3	-	37
Conn, 1963	-	-	3	2	1	-	-	6
Luetscher, 1964	-	-	-	1	-	-	-	1
Present series	-	-	3	1	1	-	-	5
Total	-	6	16	18	6	3	-	49

TABLE XVII

CONN'S SYNDROME

131 Tumours of the Adrenal Cortex: Location in Females

Number of Tumours	Site	Source				
		Review Publication Conn, 1963	Conn 1963	Luetscher 1964	Present Series	Total
Single	Right adrenal	23	4	-	5	32
	Left adrenal	53	4	4	8	69
	Bilateral	-	1	-	-	1
	Unknown	17	-	-	2	19
Multiple	Right adrenal	1	-	-	-	1
	Left adrenal	6	2	-	-	8
	Bilateral	1	-	-	-	1
	Unknown	-	-	-	-	-

TABLE XVIII

CONN'S SYNDROME

49 Tumours of the Adrenal Cortex: Location in Males

Number of Tumours	Site	Source				
		Review Publication Conn, 1963	Conn 1963	Luetscher 1964	Present Series	Total
Single	Right adrenal	11	1	-	2	14
	Left adrenal	13	3	-	2	18
	Bilateral	-	1	1	2	2
	Unknown	9	-	-	-	9
Multiple	Right adrenal	2	1	-	1	4
	Left adrenal	1	-	-	-	1
	Bilateral	1	-	-	-	1
	Unknown	-	-	-	-	-

TABLE XVIII

CONN'S SYNDROME

49 Tumours of the Adrenal Cortex: Location in Males

Number of Tumours	Site	Source				
		Review Publication Conn, 1963	Conn 1963	Luetscher 1964	Present Series	Total
Single	Right adrenal	11	1	-	2	14
	Left adrenal	13	3	-	2	18
	Bilateral	-	1	1	2	2
	Unknown	9	-	-	-	9
Multiple	Right adrenal	2	1	-	1	4
	Left adrenal	1	-	-	-	1
	Bilateral	1	-	-	-	1
	Unknown	-	-	-	-	-

tended to weigh more than 20 g., 10 of 15 tumours of this series were less than this weight and six of them were less than 6 g. in weight. When a total of 85 tumours were examined including this series, 52 were found to weigh less than 6 g. (Table XIX).

Benign Tumours

Benign tumours are usually small in size and attached at one pole by their well formed capsules to the associated adrenal glands, the appearances of which are normal. Generally the growths are ovoid or spherical and their cut surface presents a golden yellow to yellow-brown colour. The very small tumours found in Cases 2, 5 and 7 (Table XV) are wholly contained within the associated gland and only become apparent on cutting it. Areas of necrosis and haemorrhage are noted only in the larger tumours and are relatively prominent at one pole of the lesion weighing 79 g. (Case 11, Table XV).

Several different histological cell types and patterns compose these tumours. The commonest cell type is morphologically similar to the clear cells of the zona fasciculata of the normal gland (Fig. 29). They resemble such cells in size, in their nuclear-cytoplasmic ratio and high lipid content of the cytoplasm. Stainable RNA is absent or minimal, but the Δ_5-3β-hydroxysteroid dehydrogenase is present as judged by histochemical techniques. These cells are arranged in small cords or acini which are separated

TABLE XIX

CONN'S SYNDROME

85 Tumours of the Adrenal Cortex: Weight Distribution

Source	Weight (g.)								
	< 2	2-4	4-6	6-8	8-10	10-20	20-30	30	Total
Review publication Conn, 1963	14	15	7	4	5	3	1	4	53
Conn, 1963	5	2	3	1	1	4	-	1	17
Present series	2	2	2	1	2	1	2	3	15
Total	21	19	12	6	8	8	3	8	85

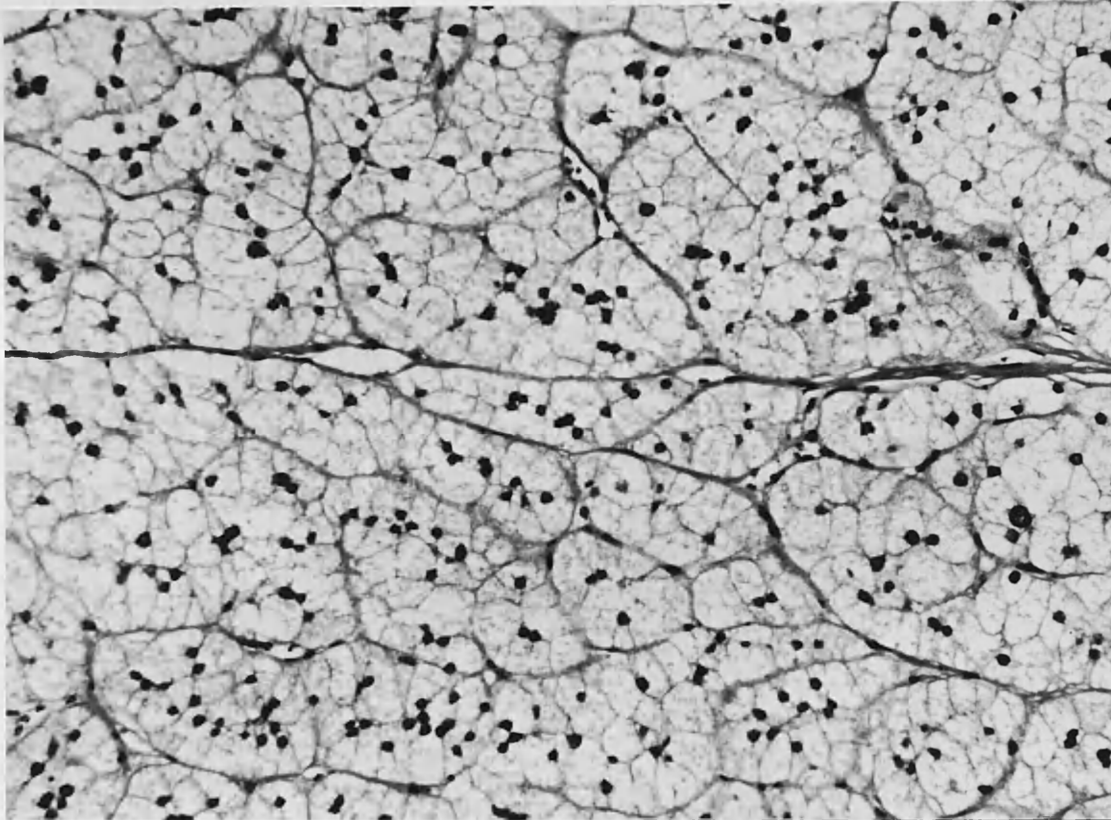


Fig. 29.- Conn's syndrome - adenoma. The component cells are similar to the clear cells of the zona fasciculata of the normal adrenal cortex. They are arranged in small alveoli or cords, separated by a fine fibrovascular stroma. Nuclear pleomorphism is minimal. H & E x240.

by fine connective tissue trabeculae in which a few capillaries can be seen (Fig. 29). The nuclei of these cells are more vesicular than those of normal clear cells and both nuclear and cellular pleomorphism can be seen in most of the tumours, but they seldom become prominent features.

Cells of the zona glomerulosa type are noted in several of the tumours, forming solid trabeculae or alveoli. They occur more commonly immediately below the tumour capsule (Fig. 30) but are also demonstrable in the more central areas of the lesion. The nuclear:cytoplasmic ratio of these cells is high and their scanty eosinophilic cytoplasm contains variable amounts of stainable lipid and RNA. Their nuclei which may show pleomorphism, are vesicular in character with prominent nucleoli.

Small groups of cells, morphologically similar to the compact cells of the zona reticularis of the normal gland are observed in a few tumours. They occur in association with the clear and glomerulosa-type cells or they may form small micro-adenomata.

Clear cells alone or in conjunction with cells of the glomerulosa and reticularis type composed all but two of the benign tumours. In four of these another morphologically distinct type of cell is apparent. Its nuclear:cytoplasmic ratio is similar to or higher than that of the zona glomerulosa, but its cytoplasm is filled with lipid and similar in appearance to the cells of the

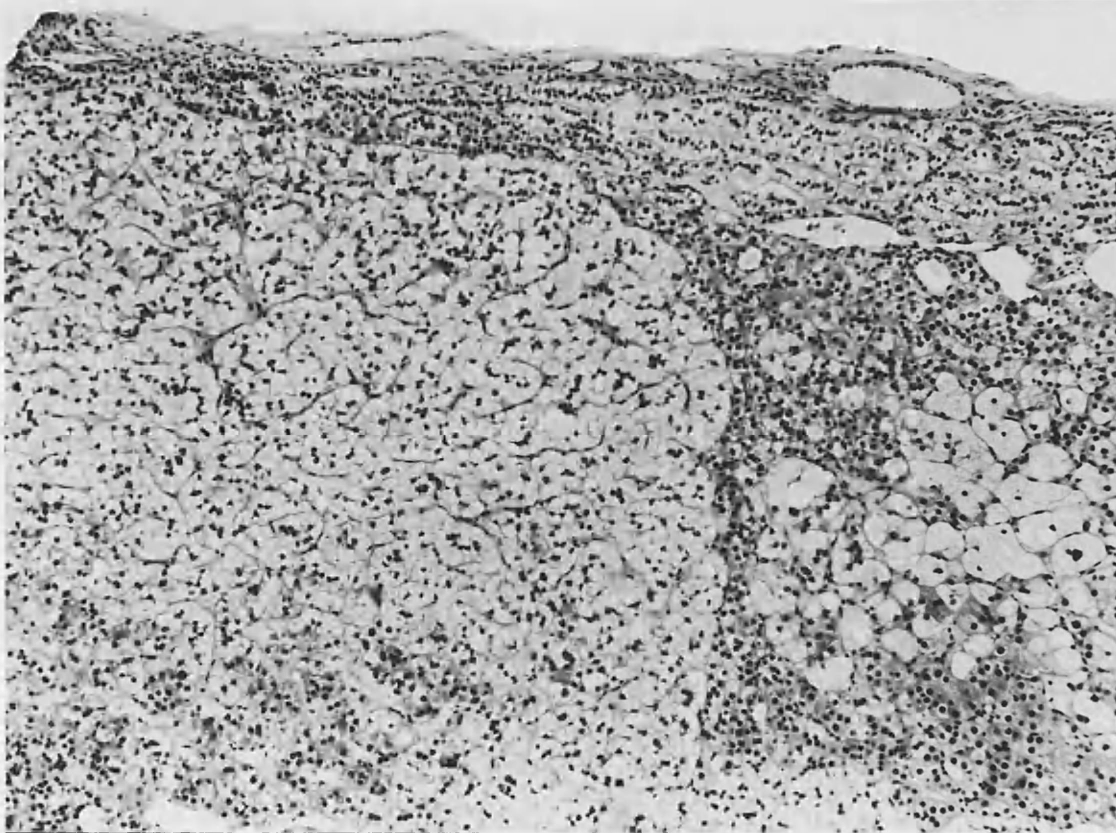


Fig. 30.- Conn's syndrome - adenoma. Three different types of cells are seen in this figure. Cells of the zona glomerulosa type are present especially subcapsularly, while large clear cells are noted on the right and the hybrid type of cell on the left of the figure. H & E x95.

the zona fasciculata. They are referred to as "hybrid cells" (Fig. 30).

Two tumours in the series (Cases 6 & 11, Table XV) are formed almost exclusively by zona glomerulosa type cells (Fig. 31), with only a few foci of clear cells. In most areas of the lesion, the arrangement of the cells is similar to the previous description, but in other zones, the trabeculae, cords or acini are noted to be separated by a myxomatous Schiff-negative stroma (Fig. 32).

These tumours possess well formed capsules from which narrow trabeculae arise and divide the lesions into a lobular pattern more especially in the larger growths.

Malignant Tumours

Both the malignant tumours are larger than the benign lesions in this series (Cases 18 & 19, Table XV) and their cut surfaces reveal a lobular pattern and areas of necrosis and haemorrhage (Fig. 33).

The morphological appearance of both tumours is very similar. The component cells are ovoid or round and large in size. They possess a finely granular vacuolated cytoplasm in which sparse amounts of lipid are demonstrable. The nuclei are large and the nuclear:cytoplasmic ratio approximates to that of the "hybrid" cell found in some benign lesions (Fig. 34). Many of the nuclei are vesicular in character and possess a finely stippled interior in

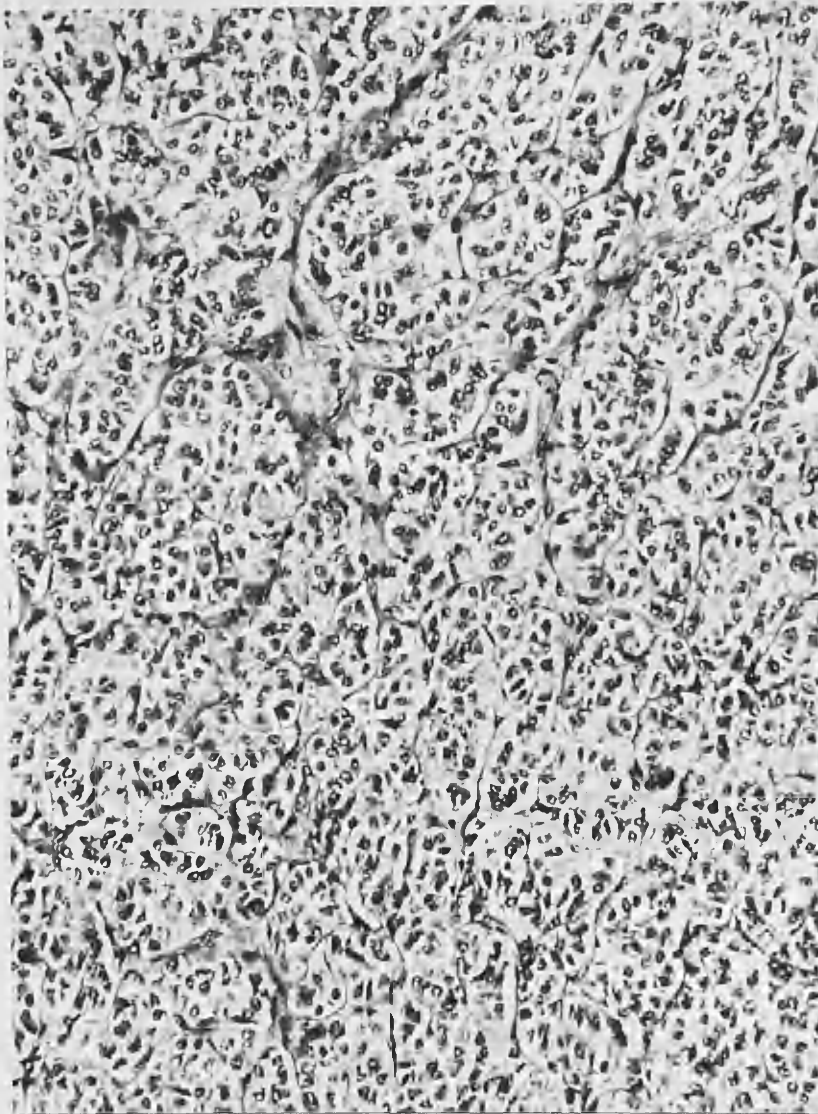


Fig. 31.- Conn's syndrome - adenoma. The component cells are of the zona glomerulosa type and they are arranged in small alveoli. Some nuclear pleomorphism can beseen. H & E x240.

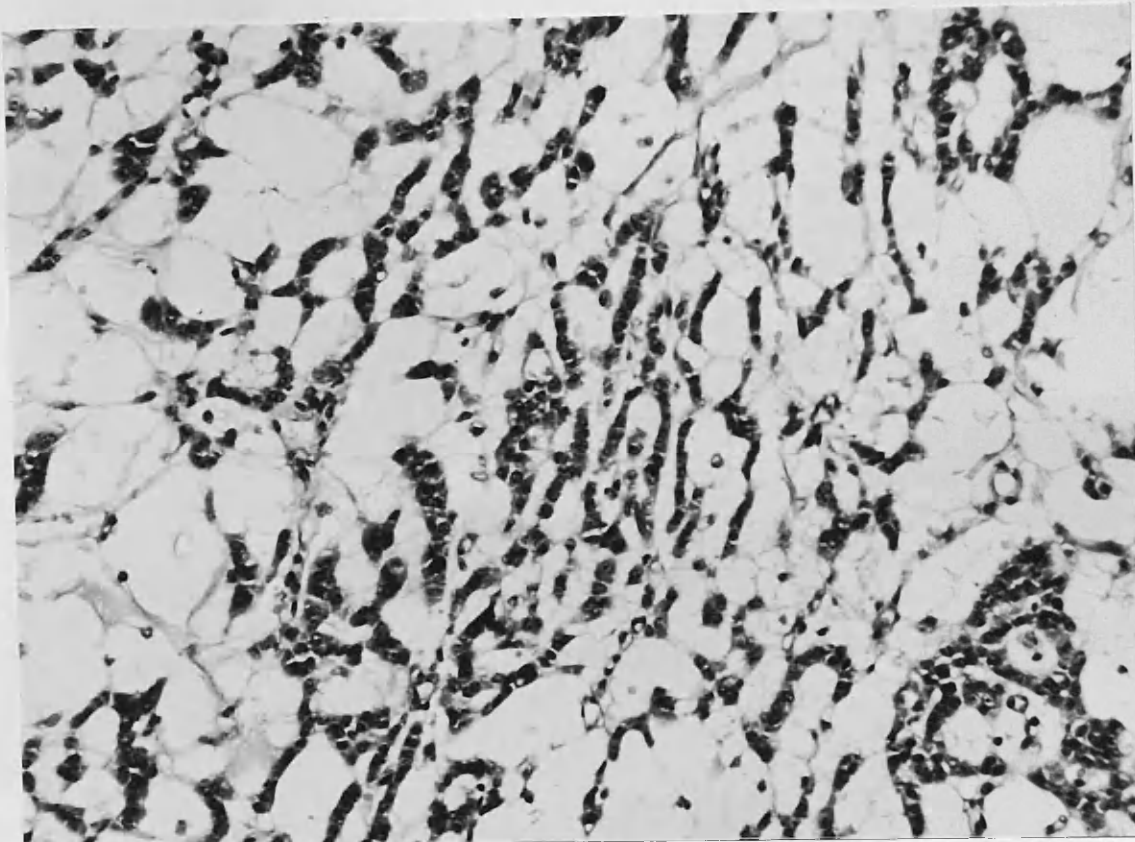


Fig. 32.- Conn's syndrome - adenoma. Cells of the zona glomerulosa type are arranged in short cords or columns, separated by an oedematous, Schiff-negative stroma. H & E x240.

Fig. 33.- Conn's syndrome - carcinoma (weight 1,400 g.). The tumour is encapsulated and divided into irregular lobules. The cut surface is soft and areas of necrosis are noted. The associated, normal sized, adrenal gland is attached at one pole of the growth.

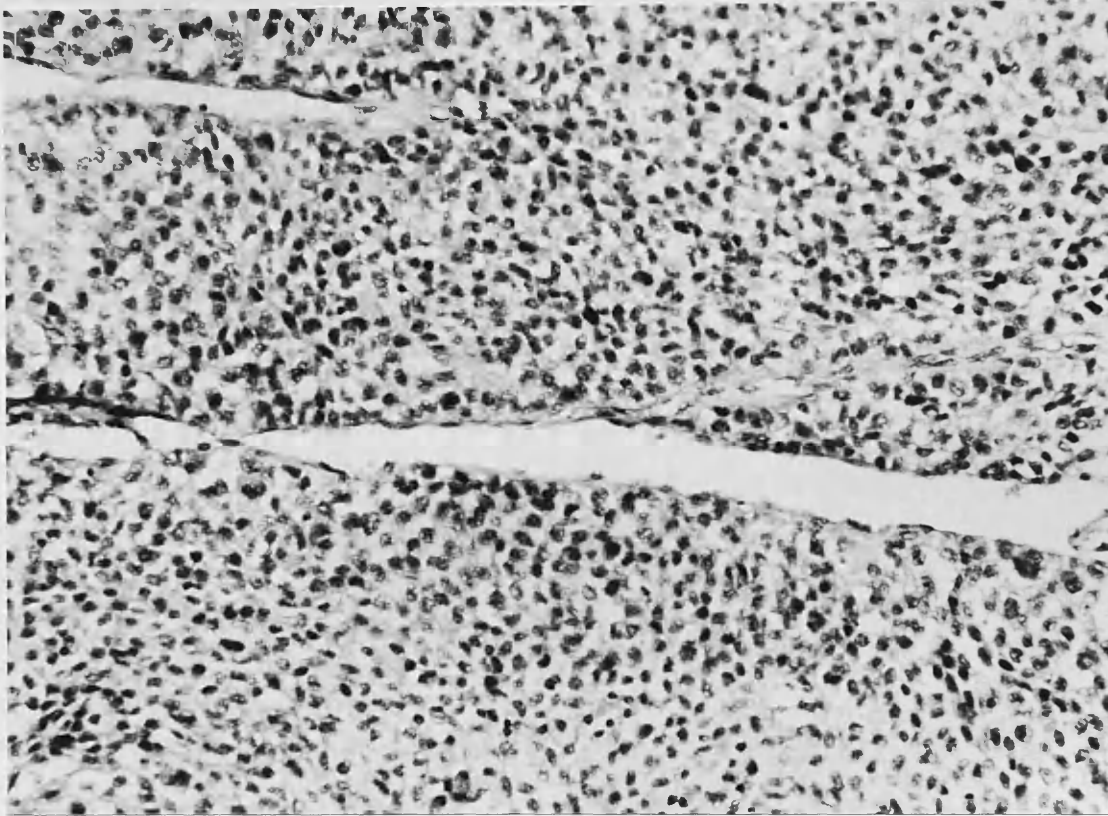


Fig. 34.- Conn's syndrome - carcinoma. The cells, which are of the "hybrid" type, are arranged in large alveoli surrounded by prominent vascular spaces. Their nuclei are vesicular in character and also show pleomorphism. H & E x240.

Fig. 35.- Conn's syndrome - adrenal gland found in association with benign or malignant tumours. The associated adrenal gland shows a prominent, broadened zona glomerulosa. The zona reticularis and fasciculata are normal in appearance. H & E x90.

and contains prominent blood filled capillaries. The zona reticularis and zona fasciculata are normal in appearance in many glands, but occasionally evidence of focal lipid depletion is seen.

DISCUSSION

It is dubious whether the incidence of the various pathological lesions associated with hypercorticalism in this survey is an accurate reflection of their true incidence. This is due perhaps to two factors. First, there is considerable selection of lesions referred to the Department of Pathology at the Glasgow Royal Infirmary, the rarer ones in particular being submitted for opinion. Secondly, with the development of adequate medical therapy for congenital adrenal hyperplasia, glands from these patients are no longer available for study except at autopsy. Thirdly, Conn's syndrome is a relatively new entity, and its true incidence in hypertension and in relation to the other adrenal disorders remains to be clarified.

The histological patterns noted in bilateral adrenocortical hyperplasia associated with Cushing's syndrome, Conn's syndrome and the adrenogenital syndrome are distinct and diagnostic, but histological differentiation of tumours causing Cushing's syndrome and both the virilising and feminizing adrenogenital syndromes is impossible. In Conn's syndrome, the diagnosis of aetiological tumours can be made on histological grounds, provided function of the tumour was proved during life.

BILATERAL ADRENOCORTICAL HYPERPLASIA

The zona glomerulosa is the site of aldosterone biosynthesis both in man (Ayres *et al.*, 1958; Siebenman, 1959) and animals (Ayres *et al.*, 1956 ; Giroud *et al.*, 1956), so that bilateral hyperplasia of this zone in cases of Conn's syndrome not due to adrenal tumours is the expected finding. Although the external appearance and weight of such glands are normal, the reports of histologically normal glands may have their foundation upon failure to appreciate the focal nature of the zona glomerulosa in the normal human adrenal cortex. In both cases where there were urinary steroid analyses to support the clinical diagnosis of Conn's syndrome, the zona glomerulosa exhibited prominent hyperplastic changes. In these cases in which estimations of the urinary aldosterone titre were not made, the hyperplasia was less marked and focal in type. This latter histological pattern is not diagnostic of Conn's syndrome; it may occur in cases of essential hypertension, especially in its malignant phase. Consequently, although the histological pattern may suggest the diagnosis of Conn's syndrome due to bilateral adrenocortical hyperplasia, evidence of raised aldosterone levels compatable with this disease must be established before the diagnosis can be made with certainty.

The aetiology of bilateral hyperplasia of the zona glomerulosa

is unknown but it has been noted to occur in two distinct groups of patients. In one, the disease occurs most frequently in young subjects especially males and is associated with malignant hypertension, while in the other, benign hypertension is found in adults particularly females between the ages of 30 and 50 years. The disease has been reported to be due to both hyperplastic and normal glands in both groups (von Buchem et al., 1956; Holten & Petersen, 1956; Bartter & Biglieri, 1958; Conn & Conn, 1961). Bilateral adrenalectomy fails to cure the hypertension in the older patients, but succeeds in the younger age group. However, the latter still exhibit hypersensitivity to mineralocorticoids followi operation (Conn et al., 1964) and the suggestion has been made that their disease is due to a congenital anomaly of the renal juxtaglomerular apparatus (Conn & Conn, 1961).

The hyperplasia of the zona glomerulosa found in association with adrenal tumours causing Conn's syndrome would appear to be paradoxical. It may be related, however, to the potassium loading which these patients receive pre-operatively to raise their serum levels to normal.

The changes which occur in the adrenal cortex in response to ACTH depend upon its dose and type (Symington et al., 1956; Studzinski et al., 1963). In bilateral adrenocortical hyperplasia associated with Cushing's syndrome and the adrenogenital syndrome,

the morphological changes are consistent with an elevation in the circulating ACTH concentration. The normal plasma level of ACTH was found to be 0.75 milliunits/100 ml. by Davies and her colleagues (1960) and it was found to be raised to between 2 and 3 milliunits/100 ml. in Cushing's syndrome due to bilateral adrenal hyperplasia (Davies _et al._, 1960). Using the same method, a value of 5.71 milliunits/100 ml. has been reported in a case of congenital adrenal hyperplasia (Hamilton & Brush, 1964). The compact cell zone found in the hyperplastic adrenals is not as broad in Cushing's syndrome as it is in the adrenogenital syndrome. Thus the appearances are consistent with those plasma ACTH levels.

In the adrenogenital syndrome, this increased secretion of ACTH results from a reduced level of circulating cortisol, the biosynthesis of which is impaired by a deficiency in the adrenal enzyme systems (Jailer _et al._, 1955; Eberlein & Bongiovanni, 1956; Bongiovanni, 1958; Bongiovanni, 1961). The low cortisol level releases the anterior pituitary from its inhibitory effect. The elevated ACTH values in bilateral adrenal hyperplasia in Cushing's syndrome, not due to pituitary tumours, would appear to be a primary pituitary or hypothalamic effect, the latter mediated perhaps by the corticotrophin releasing factor (CRF). The hypothalamo-pituitary axis is deranged in this disorder not only in its increased ACTH secretion, but also in its unresponsiveness to

suppression by normal levels of blood cortisol, the feed-back mechanism working at an abnormally high level (Williams et al., 1961) so that large doses of suppressant cortisol analogues are required (Liddle, 1960). A loss in the diurnal rhythm of ACTH secretion has been postulated (Williams et al., 1961). Pituitary tumours cause Cushing's syndrome due to their increased secretion of ACTH but none of the present patients have been found to possess such lesions either before or after therapeutic adrenalectomy.

It may be possible to explain the occurrence of glands morphologically hyperplastic but of normal weight in Cushing's syndrome on the basis of the secretion of different types of ACTH. An adrenal weight factor, described by Stacke-Dunn and Young (1951) has been demonstrated in the plasma in some patients with Cushing's syndrome (Christy et al., 1957). From their studies with crude and purified forms of ACTH, Studzinski and his colleagues (1963) showed that the degree of hyperplasia occurring in normal glands removed from patients with metastatic breast carcinoma was related to the type of ACTH administered. Using the crude preparation containing both a steroidogenic and a weight factor, a 115% increase in adrenal weight was achieved, whereas little or no increase in weight occurred with the more highly purified ACTH preparations, equal in steroidogenic potency. Consequently, both factors

may be elevated in Cushing's syndrome when the gland weighs over 6.0 g., while only the steroidogenic factor is elevated in patients with the smaller glands. The occurrence of a marked increase in weight and hypertrophic cellular changes in Cushing's syndrome due to bilateral hyperplasia may be due to the predominance of the weight factor, as similar weight and morphological alterations were noted in response to crude ACTH preparations (Symington *et al*., 1956).

The increased secretion of ACTH has also been implicated in the causation of bilateral adenomatous hyperplasia in Cushing's syndrome (Kirschner *et al*., 1964). As two of the four previously reported patients with this condition (Cases 9 & 12, Table VIII) suffered from the disease for a long time prior to treatment, the suggestion was made that prolonged exposure to increased concentrations of ACTH might be responsible. However, this is not apparent in the patients of the present series, particularly the three month old female child (Case 8, Table XIII) and some other factor must be responsible for the change.

The importance of this disorder lies in the need to differentia it from an adenoma causing Cushing's syndrome. All functioning adrenocortical tumours causing Cushing's syndrome are found in conjunction with atrophy of the associated and contralateral glands, whereas adenomatous hyperplasia is associated with hyperplasia of

the non-adenomatous areas of the cortex. Moreover as adenomatous hyperplasia is a bilateral condition, both glands require surgical treatment, whereas removal of a unilateral functioning tumour is curative.

Further difficulty occurs in the differentiation of adenomatous hyperplasia from an adenoma as the former entity can simulate a benign functioning tumour in its response to the infusion of ACTH and suppression with dexamethasone. An exaggerated response to ACTH was found in five cases of adenomatous hyperplasia of either the present or previously reported cases (Table VIII) and failure to suppress with dexamethasone or a similar pharmacological agent in six. These responses can also be obtained in cases of Cushing's syndrome caused by adenomas (Liddle, 1960) so that some degree of autonomy is possessed by either the adenomatous nodule or the hyperplastic gland. It is presumed that the response to ACTH is elicited due to their content clear cells. Thus diagnostic confusion may occur leading to inadequate surgical treatment if this feature is not known.

Recent studies in Cushing's syndrome found in association with tumours of "non-endocrine" tissues have suggested that ACTH or a polypeptide with similar biological and chemical properties is elaborated by these tumours and is responsible for the changes in structure and function of the adrenal cortex (Meador et al.,

1962; Nichols et al., 1962; Liddle et al., 1963; Marks et al., 1963; Scholz et al., 1963; Hallwright et al., 1964). Since the first published case of this association (Brown, 1928), many further reports have confirmed its existence. Fifty-eight cases, mostly due to oat cell carcinomas of bronchus were reviewed in 1961 (Riggs & Sprague, 1961). Carcinomas of the thymus were the first type of tumour to establish the relationship with certainty (Leyton et al., 1931; Duguid & Kennedy, 1933; Hubble, 1949; Sprague, 1950; Soutter et al., 1957; McCullagh & Tretbar, 1958; Scholz & Bahn, 1959; Camus et al., 1961; Mucic & Arsenijevic, 1963). Of 13 cases of the syndrome reported by Liddle and his associates (1963), nine occurred in patients with oat cell carcinomas of the bronchus. Carcinomas of the islet cells of the pancreas, the mediastinum and the parotid and a phaeochromocytoma were found in each of the remaining four cases. The association with tumours of the pancreatic endocrine (del Castillo et al., 1950; Howard et al., 1950; Rosenberg, 1956; Balls et al., 1959; Farrant & Insley, 1960; Meador et al., 1962) and exocrine tissues (McLetchie & Scott, 1943; Crooke, 1946; Peart et al., 1963) has been noted by other workers and argentaffin tumours of the pancreas (Hallwright et al., 1964) and bronchus (Escovitz & Reingold, 1961) have been found in causal relationship.

While a definite association exists between those entities

and adrenal hyperplasia, the co-existence of the syndrome in patients with carcinomas of the colon (Warren, 1945), prostate (Webster et al., 1959), gall-bladder (Brickner et al., 1961), testis (Di Ferranti etal., 1951) and ovary (Parsons & Rigby, 1958) may be fortuitous.

Bronchogenic carcinoma was found in each of the 8 cases of this series. The adrenal glands tended to be larger and heavier with the exception of Case 8 (Table VIII) than those of other cases of bilateral hyperplasia in Cushing's syndrome. The histological pattern resembles the hyperplasia found in congenital adrenal hyperplasia with the exception that clear cells cap the columns of compactcells in some glands. These clear cells may be responsible for the positive response to the infusion of ACTH noted in some patients (Werk & Sholeton, 1960; Christy, 1961; Meador et al., 1962; Marks et al., 1963). In their absence, no response to ACTH is obtained (Meador et al., 1962; Marks et al., 1963). Of interest is the lack of suppression of the secretion of corticosteroid by dexamethasone (Meador et al., 1962; Liddle et al., 1963; Marks et al., 1963; Friedman et al., 1965). These tests serve to differentiate the disease from bilateral hyperplasia, but fail to do so in patients with bilateral adenomatous hyperplasia or a functioning cortical adenoma.

The ninth case (Case 9, Table VII) in this series was

associated with a malignant argentaffin tumour of the stomach. The morphological picture of both glands was in keeping with bilateral hyperplasia due to an hypothalamo-pituitary defect of ACTH secretion rather than to the secretion of ACTH by the tumour itself.

Clinical evidence of Cushing's syndrome may be absent (Hudson & Evans, 1962; Marks et al., 1963; Meador et al., 1963; Scholz et al., 1963; Cohen et al., 1964; Friedman et al., 1965) and only the steroid biochemical aberrations present. This was seen in one of the patients of this series (Case 8, Table VII) but hypokalaemic alkalosis and pigmentation often serve to attract attention. The former is due to the increased cortisol levels (Christy & Laragye, 1961) and not to an increase in aldosterone secretion (Scholz et al., 1963) while the latter is caused by the secretion of MSH by some of these primary tumours (Hallwright et al., 1964).

In 16 cases, the presence of ACTH or a similar polypeptide has been demonstrated not only in the primary but also the secondary tumours (Meador et al., 1962; Nichols et al., 1962; Liddle et al., 1963; Marks et al., 1963; Hallwright et al., 1964). Increased plasma ACTH levels (Liddle et al., 1963; Scholz et al., 1963) and a low concentration in the adenohypophysis (Nichols et al., 1962; Liddle et al., 1963; Marks et al., 1963) have also

been described. These findings could be interpreted as being the result of increased ACTH production by the adenohypophysis and its subsequent storage by the tumour. However, the clinical and biochemical manifestations of Cushing's syndrome recede following surgical removal of the primary tumours (Liddle et al., 1963; Micic & Arsenijevic, 1963) so that these tumours appear to be responsible for the elaboration and secretion of ACTH or a structurally similar protein (Liddle et al., 1963). The presence of metastases in the median eminence of the brain was shown in Case 7 (Table VII), but not in a further two cases (Cases 2 & 3, Table VIII) (Dobbie, personal communication). Although this may represent another method whereby increased amounts of ACTH may be produced through the agency of CRF, its absence in other cases makes it an unlikely cause.

Two further aspects of adrenocortical hyperplasia merit consideration. Remissions occur rarely in Cushing's syndrome, but were noted in two cases of this series, one of which was induced by radiotherapy. Lipid infiltration of the zona reticularis was noted to occur. Lipid laden cells occupying the site of the zona reticularis with hyperplastic changes outwith this zone was observed in two patients in whom remission with o'p'DDD was obtained, but a relapse developed subsequently.

The hyperplasia of the zona glomerulosa noted in five of

the six cases of congenital adrenal hyperplasia probably represents a reactive change as the biosynthesis of aldosterone requires the enzymes found to be deficient in this syndrome. However, the zona glomerulosa has been noted to be absent (Blackman, 1946; Lewis et al., 1950) or hyperplastic (Bernhiem et al., 1954) in other glands. Normal aldosterone levels have been found in cases of congenital adrenal hyperplasia due to a 21-hydroxylase defect unassociated with salt-loss, while low levels occurred in association with salt loss (Degenhart et al., 1965). The relation of these values to the appearance of the zona glomerulosa remains to be elucidated.

An appreciation of the morphological changes which occur in the human adrenal cortex due to stress or the administration of ACTH, enables a correct interpretation of the distinctive hyperplastic patterns found in Cushing's and the adrenogenital syndromes. As the zona glomerulosa is the site of aldosterone biosynthesis, only this zone is affected in Conn's syndrome due to bilateral adrenocortical hyperplasia.

TUMOURS OF THE ADRENAL CORTEX

If the concept is accepted of the compact cell of the zona reticularis being responsible for most of the production of corticosteroids with the exception of aldosterone, the androgens

and the oestrogens required by the body from day to day, then it is possible to understand why the morphological appearance of tumours causing Cushing's syndrome and the adrenogenital syndrome are identical. Since both benign and malignant tumours consist exclusively, or almost exclusively, of compact cells, it is not possible on histological grounds alone to distinguish between those of differing endocrine capacity. The exception to this is the small tumour, usually weighing less than 70 g., associated with the _pure_ form of Cushing's syndrome in which admixtures of clear and compact cells occur, the former predominating.

Small cortical adenomas are commonly found at autopsy, the majority being apparently non-functioning _in vivo_. Histological examination gives no information on the functional capacity of these tumours and both they and the uncommon non-functioning carcinoma may be composed of compact or clear cells. In the latter event, distinction from the tumours of Conn's syndrome may be impossible. Some of the non-functioning clear cell adenomas respond to the stress of dying with transformation of some of the clear cells to compact cells. Their resemblance to the small benign tumours causing Cushing's syndrome is readily apparent.

A suggestion has been made recently, that an enzyme defect, possibly the Δ_5-3β-hydroxysteroid dehydrogenase, is present in some non-functioning tumours, the reason for the lack of

symptomatology being due to the failure fo form physiologically active steroids (Fukushima & Gallagher, 1963).

The incidence of adrenocortical tumours in Cushing's syndrome would appear to be of the order of 25% (Table II) benign and malignant tumours occurring almost equally. An accurate assessment of the incidence of malignancy is difficult due to the different criteria used by different investigators. Benign tumours have been reported more frequently in some reports (Wilkins, 1948; Cahill & Melicow, 1950; Poutasse & Higgins, 1952) and malignant lesions in others (Weinberg, 1941; Markes et al., 1940; Soffer et al., 1961). From the 117 cases of Cushing's syndrome due to tumours collected by Rapaport and his colleagues (1952), 73 were stated to be malignant and 35 benign. In 9 cases, the pathological diagnosis was of "adrenal tumour". Tumours occurred more frequently in females and especially after the age of 12 years (Rapaport et al., 1952; Heinbecker et al., 1957). The present series conforms to these observations.

The adrenogenital syndrome due to virilising tumours occurs more often below the age of 12 years, but like Cushing's syndrome more commonly in females (Rapaport et al., 1952; Heinbecker et al., 1957). These frequencies occur in this series. As in Cushing's syndrome, the incidence of malignancy in tumours causing the adrenogenital syndrome is difficult to guage, but of the 71 cases

reported by Rapaport and his co-workers (1952) 46 were malignant.

Feminizing tumours of the adrenal cortex occur most frequently between the ages of 25 and 50 years (Gabrilove et al., 1965), although they have been found in children from the age of 5 years (Wilkins, 1948; Snaith, 1958) and in adults up to the age of 66 years (Case Records, Massachusetts General Hospital, 1955). Of the 52 feminizing tumours reviewed by Gabrilove and his colleagues (1965), 41 were diagnosed as carcinomas of which 30 subsequently developed metastases. Of the 10 tumours diagnosed as adenomas, only a few of the patients have been followed for sufficiently long periods for this diagnosis to be accepted. Those include the cases reported by Ostergaard (1947), Wilkins (1948), Mortensen and Murphy (1951), Dostal (1955), Mosier and Goodwin (1961) and Dempsey and Hill (1963). The follow-up periods range from 2 to 14 years. Long periods of reappraisal are essential before a benign tumour can be established, as the development of metastases has been known to be delayed for as long as seven years (Roholm and Teilum, 1942; Kerr & Gordan, 1952; Myhre, 1952; Dohan et al., 1953; Landau et al., 1954; Wallach et al., 1957).

Feminizing tumours vary greatly in weight from 10 g. (Snaith, 1958) to over 2 kilogrammes (Roholm & Teilum, 1942; Myhre, 1952; Higgins et al., 1956). While benign tumours tend to be lighter and smaller (Mortensen & Murphy, 1951; Dostal, 1955;

Snaith, 1958; Mosier & Goodwin, 1961), a considerable overlap occurs (Hall, 1930; Luft & Sjögren, 1949; Dohan _et al._, 1953; Fontaine _et al._, 1954; Dempsey & Hill, 1963). This has also been observed in virilising adrenal tumours (Fordyce & Evans, 1929).

Consequently all feminizing tumours must be regarded with suspicion. Both tumours in this series showed similar histological patterns, and one was found to have metastasised (Case 1, Table XIV) the other lesion must be regarded as possibly malignant.

Conn's syndrome is most commonly due to tumours, the majority of which are benign (Conn, 1963). They occur particularly in adult life, between the ages of 31 and 50 years. The present series corresponds closely with the other reported cases and the morphological appearance of the tumours present distinct and diagnostic patterns. However, tumours which are composed of only clear cells, although they often exhibit pleomorphism, cannot be distinguished histologically from the non-functioning adenomas composed of clear cells unless _in vivo_ function has been demonstrated

The relative infrequency of cells of the zona glomerulosa type is difficult to understand as these tumours have been found to be capable of aldosterone biosynthesis _in vitro_ (Ayres _et al._, 1958; Bailey _et al._, 1960; Davignon _et al._, 1961; Pasqualini, 1964; Fazekas & Webb, 1965). Moreover they can also form cortisol and corticosterone (Davignon _et al._, 1961; Louis & Conn, 1961;

Brode et al., 1962; Fazekas & Webb, 1965).

Zona glomerulosa cells of the normal adrenal gland lack the 17α-hydroxylase system but possess an "18-oxidase" required for the production of aldosterone, whereas the cells of the normal zona fasciculata lack the "18-oxidase", but possess the 17α-hydroxylase required for the formation of cortisol. Brode and his colleagues (1962) studied three tumours causing Conn's syndrome and by histological control of the incubated tissue noted that while the cells of glomerulosa type produced similar steroids as normal zona glomerulosa cells, the clear cells of the tumour produced steroids characteristic of both normal clear and glomerulosa cells. For this reason these clear cells are referred to as "hybrids". The subsequent finding in other tumours of morphologically "hybrid" cells confirms the biochemical observation. Consequently, all the clear cells of these tumours appear to be hybrid in type as judged biochemically and this helps to explain why other workers have also noted the predominant or sole content of clear cells in aldosterone producing tumours (Ross, 1959; Bailey et al., 1960; Conn& Conn, 1961; Davignon et al., 1961; Conn, 1963).

The histological patterns noted in the tumours removed from cases 6 and 11 (Table XV) present a diagnostic problem. The weight of the tumour removed from Case 6 is unknown, but the lesion from

Case 11 weighed 79 g. This is more in keeping with malignant than benign tumours. The histological pattern in both tumours which consisted of cords of zona glomerulosa type cells separated b an oedematous stroma, is similar to the histological description of the malignant tumour described by Foye and Feichtmeir (1955). However, both patients in this series are alive and well 4 and 5 years post-operatively.

Only five malignant tumours have been reported previously (Foye & Feichtmeir, 1955; Brooks et al., 1957; Zimmerman et al., 1959; Conn et al., 1964; Luetscher, 1964). Case 18 (Table XV) is the patient reported by Brooks and his colleagues (1957). Case 19 (Table XV) presented with signs and symptoms of both Conn's syndrome and Cushing's syndrome. These two tumours are similar. The cell type is compatible with the appearance of the "hybrid" cell, and this accords with the finding not only of elevated urinary aldosterone levels, but also raised urinary 17-hydroxy, 17-ketogenic and 17-ketosteroids (Foye & Feichtmeir, 1955; Brooks et al., 1957; Case 19, Table XV). The histological appearance of these two growths is distinctive and allows diagnosis of malignant tumours causing Conn's syndrome but in view of the histological pattern noted by Foye and Feichtmeir (1955), not all malignant tumours in this group are similar morphologically

As with other endocrine tumours, it is often difficult to

determine whether adrenocortical tumours are benign or malignant. This has been observed by other workers particularly Symington and Jeffries (1962) and Lipsett and his colleagues (1963). The usual criteria of malignancy cannot be applied. Mitotic figures can be observed not only in malignant tumours but also in benign lesions and, furthermore, they may be absent in proved metastasing carcinomas (Symington & Jeffries, 1962; Lipsett et al., 1963). Invasion of the tumour capsule has been proposed as being most suggestive of carcinoma, but this feature and apparent venous invasion have been found in non-malignant endocrine tumours. Importance has been attached to the size of the tumour, but a considerable overlap in weight has been observed not only in this series but also by others (Fordyce & Evans, 1929; Hall, 1930; Luft & Sjögren, 1949; Rapaport et al., 1952; Dempsey & Hill, 1963). Thus, although extremes in weight are helpful, the larger tumours being more likely to be malignant, this cannot be used as a reliabl criterion.

Several features which have been found to occur in the maligna tumours of this series appear to be relatively reliable. The presence of enlarged, vesicular nuclei with prominent nucleoli was noted in every malignant tumour and was also present in the metastases examined at autopsy. When extensive areas of necrosis involved the tumours, the lesion almost always behaved in a maligna

fashion. When marked nuclear pleomorphism is observed in association with enlarged vesicular nuclei and extensive necrosis, this feature would appear to serve as a guide to malignancy. However, if pleomorphism is found alone, then the tumour can behave as a benign or a malignant growth.

The finding of enlarged vesicular nuclei with or without pleomorphism and with extensive areas of cellular necrosis is the most reliable index of malignancy available at present. These features can occur alone or in association with the usual criteria for the histological diagnosis of malignancy. Nevertheless, the only absolute criterion of malignancy is the detection of distant metastases, so that careful follow-up over long periods is required in every instance.

SUMMARY

A review of 122 cases of hypercorticalism has been presented. The pathological features of the lesions associated with the various syndromes have been submitted to a critical reappraisal based upon the observations of the effects of stress and ACTH upon the normal human adrenal cortex.

Cushing's syndrome was the most frequent entity and bilateral adrenocortical hyperplasia its most common pathology. Almost all patients suffering from Conn's syndrome were found to have tumours of the adrenal cortex. Similarly the adrenogenital syndrome was caused principally by adrenal tumours.

The pathological problems associated with the diagnosis of these conditions have been discussed in detail and the difficulties encountered in deciding whether a particular tumour was benign or malignant have been outlined.

P A R T I I

THE IN VITRO BIOSYNTHESIS OF ADRENAL ANDROGENS

INTRODUCTION

GENESIS OF THE SECRETIONS OF THE ADRENAL CORTEX

Historical Account.

The earliest demonstrations of the vital nature of the adrenal cortex were made by Rogoff and Stewart (1927) and Swingle and Pfiffner (1930) in the United States. Following bilateral adrenalectomy in dogs and cats respectively, they found that extracts of the adrenal glands were capable of maintaining the animals in a relatively normal state of health for long periods whereas the control untreated animals died within seven to ten days. This "life-maintaining" function was thought to be due to a unitary type of adrenocortical secretion, named "cortin" (Hartman *et al.*, 1928), which was found to reside in the lipid soluble fraction of the extracts (Swingle & Pfiffner, 1931).

Systematic chemical studies of adrenocortical extracts were undertaken by three groups of workers in an attempt to isolate the active principle. Four crystalline compounds were found by Wintersteiner and Pfiffner (1936) but none appeared to possess any biological activity. Also in the United States, Kendall and his associates isolated five compounds, one of which possessed biological activity (Mason *et al.*, 1936a, 1936b). This substance referred to as Kendall's compound E, was subsequently shown to be cortisone. By 1936, Reichstein, working in Switzerland, had

isolated six compounds from extracts of beef adrenal glands and had made preliminary observations upon their chemical nature (Reichstein, 1936).

These isolation studies culminated in the structural identification of twenty-eight crystalline steroidal substances in 1943 (Reichstein & Shoppee, 1943), so ending the confusion that existed due to their designation by different letters of the alphabet. Cortisone, for example, had been found by all three groups of workers and was variously referred to as Kendall's compound E, Wintersteiner's compound F and Reichstein's substance FA.

The magnitude of the problem in identifying these steroids can only be appreciated when it is realised that from the extracts of both adrenal glands, of 20,000 cattle, 200 mg. of cortisone (Reichstein & von Euw, 1938), 34 mg. of cortisol (Reichstein, 1937a, 1937b), 300 mg. of corticosterone (Kendall, 1937) and 6 mg. each of 11-deoxycortisol and 11-dehydrocorticosterone (Reichstein & von Euw, 1938) only were isolated.

From the preliminary extracts, crystalline steroids were found which possessed progestational, androgenic and oestrogenic biological activity (Reichstein & Shoppee, 1943). In addition, a non-crystalline portion of the extract, called the "amorphous fraction", was found to contain the majority of the biological

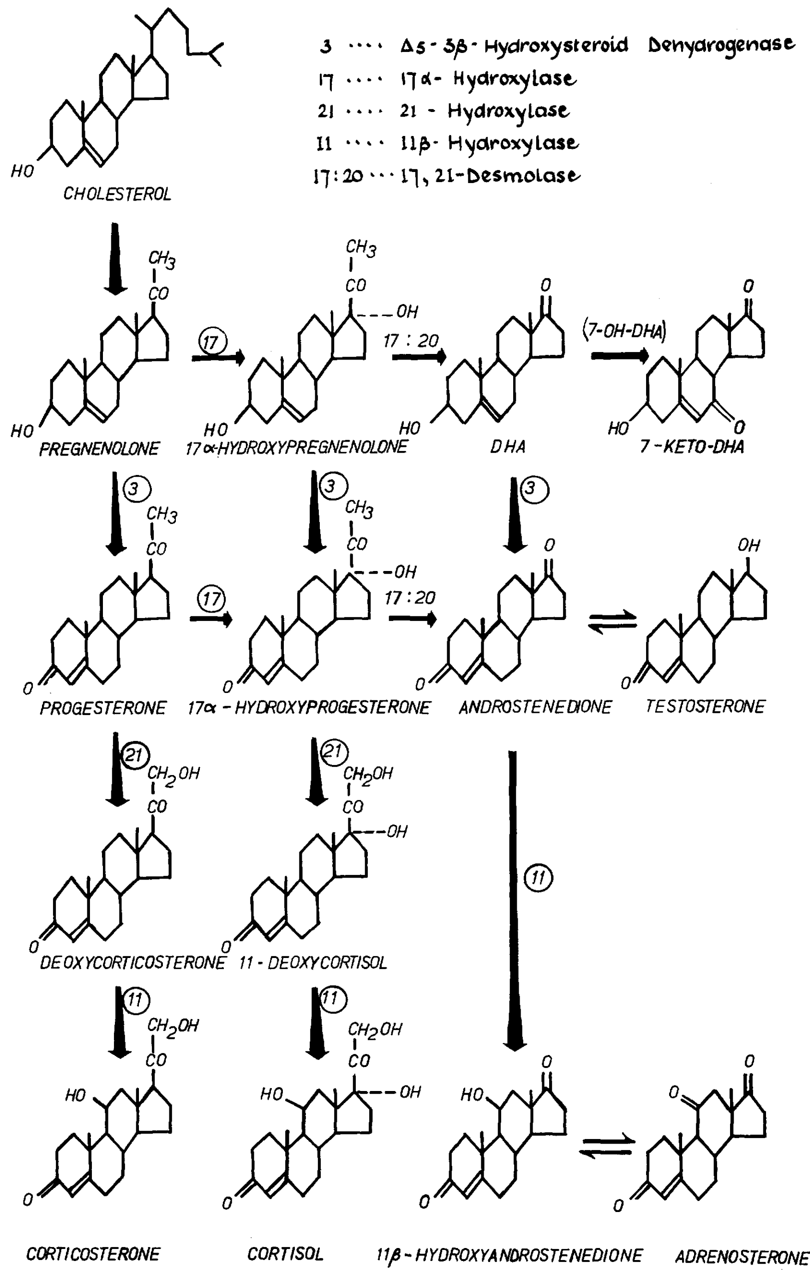
activity required to maintain life in adrenalectomised animals (Kendall, 1942; Reichstein & Shoppee, 1943; Kendall, 1948). From this fraction, the steroid hormone, aldosterone, was eventually recovered and identified (Simpson *et al.*, 1952; 1953).

Vogt (1943), in her classical experiments with dogs, demonstrated that the steroids present in adrenal venous blood were in higher concentrations than could be isolated from the cortex of these animals. This was a landmark in the investigation of the products of the adrenal cortex, but no detailed follow-up of these observations was possible at that time due to the inadequate techniques available for microchemical work. With the development of paper chromatographic systems by Zaffaroni and his colleagues (Zaffaroni & Burton, 1951; Burton *et al.*, 1951) and by Bush (1952) a significant new research field was pioneered. These techniques together with the development of infra-red spectrophotometry (Jones & Dobriner, 1949) and sulphuric acid chromagens (Zaffaroni, 1950) for the identification of steroids led to an upsurge in the investigation of the adrenal cortex which was necessitated by the discovery of the possible therapeutic application of cortisone (Hench *et al.*, 1948).

Under the direction of Hechter and Pincus working in the Worcester Foundation for Experimental Biology, the isolated beef adrenal gland was perfused *in vitro* and from these experiments,

FIG. 36

MAIN PATHWAYS OF ADRENAL STEROID BIOSYNTHESIS



Following the in vitro perfusion of bovine adrenal glands with ^{14}C labelled acetate and cholesterol, Zaffaroni (1951) and Hechter and their colleagues (1953) isolated both radioactive corticosterone and cortisol. This has been confirmed using different in vitro techniques for the incubation of other animal adrenal glands (Haines, 1952; Heard et al., 1954; Bligh et al., 1955). Cholesterol was the more efficient substrate for steroid hormone production in most of these experiments, indicating its more proximal position in the biosynthetic pathway from acetate. However, from a comparison of the rates of incorporation of labelled acetate and cholesterol into cortisol and corticosterone by perfused bovine adrenal glands in the presence of ACTH, a pathway for the formation of these hormones seemed to exist which bypassed cholesterol (Hechter et al., 1953). This was confirmed in the hog when radioactive corticosteroids were isolated following in vitro incubation with acetate-1-^{14}C but the tissue cholesterol isolated possessed no radioactivity (Bligh et al., 1955).

The use of the in vitro perfusion technique of cattle adrenal glands was largely responsible for the delineation of the biosynthetic pathways from cholesterol. Using pregnenolone, progesterone, 17α-hydroxyprogesterone and 11-deoxycortisol as substrates, the isolation of cortisol was achieved in each experimen (Hechter et al., 1951; Levy et al., 1953; Levy, 1954). Similarly

corticosterone was found to be formed from pregnenolone, progesterone and deoxycorticosterone (Hechter _et al_., 1951; Hechter & Pincus, 1954). Similar findings were reported for the biosynthesis of cortisol and corticosterone using homogenates of animal adrenal glands (Plager & Samuels, 1952; Plager & Samuels, 1953; Dorfman _et al_., 1953; Samuels, 1953).

Cortisol, corticosterone and cortisone have been extracted from human adrenal glands (Neher, 1958) and shown to be produced _in vitro_ from endogenous substrates (Cooper _et al_., 1958; Dyrenfurth _et al_., 1960). Using suitable steroid precursors, normal and hyperplastic human adrenal glands and neoplastic human adrenal tissue were found to be capable of the oxidation of the hydroxl group at position 3, and hydroxylation at positions 17, 21 and 11 (Hayano & Dorfman, 1952; Lombardo _et al_., 1956; Lombardo & Hudson, 1959; Goldstein _et al_., 1960; Roversi _et al_., 1963). _In vivo_, the administration of cholesterol, doubly labelled with ^{14}C and tritium, resulted in the isolation of cortisol and its metabolites in urine as well as some 17-ketosteroids labelled with the same ratio of $^{14}C:^{3}H$ as the administered substrate (Werbin & Le Roy, 1954; Werbin & Le Roy, 1955).

Cholesterol has been converted to pregnenolone and progesterone _in vitro_ (Saba _et al_., 1954; Lynn _et al_., 1955; Staple _et al_., 1956). 20α-hydroxycholesterol was thought to be the intermediary

(Lynn *et al.*, 1955; Solomon *et al.*, 1956) and ACTH has been shown to have a stimulatory effect upon the utilization of not only cholesterol but also 20α-hydroxycholesterol in cell free enzyme preparations of bovine adrenal homogenates (Shimizu, 1963). Δ_4-cholestenone has been found not to serve as a precursor of the steroid hormones (Hechter *et al.*, 1953; Kowal *et al.*, 1964a).

Although the reaction steps are not elucidated in full, cholesterol is established as a major precursor of the hormones in both man and animals. Following the formation of progesterone, it has been postulated that an orderly sequence of hydroxylations of progesterone occur at positions 17, 21 and 11 to form cortisol or positions 21 and 11 to form corticosterone (Hechter & Pincus, 1954; Samuels, 1960). This appears to be true but other alternative routes for the formation of cortisol have been demonstrated recently.

Cortisol is the major steroid produced by both bovine and human adrenal glands (Pincus & Romanoff, 1953), yet using progesterone labelled with ^{14}C, a greater net synthesis of corticosterone than cortisol was achieved (Eichhorn & Hechter, 1957; Berliner *et al.*, 1958) and the specific activity of the isolated corticosterone extracted after the incubation of human adrenal glands with progesterone was higher than that of cortisol (Mulrow & Cohn, 1961). This suggested the existence of a

pathway for the formation of cortisol which did not include progesterone. Pregnenolone was found to be converted to 17α-hydroxy pregnenolone, which was subsequently oxidised to yield 17α-hydroxyprogesterone (Cox, 1960) and it was noted that 17α-hydroxypregnenolone served as a substrate for the production of cortisol by normal human , rat and guinea-pig adrenal glands (Lipsett & Hökfelt, 1961) and by hyperplastic human adrenal glands removed from patients with Cushing's syndrome (Mulrow <u>et al</u>., 1962). The <u>in vitro</u> incubation of a tumour causing Cushing's syndrome was shown to utilize 17α-hydroxypregnenolone more efficiently than progesterone for the formation of cortisol (Weliky & Engel, 1962). Pregnenolone was converted <u>via</u> 17α-hydroxypregnenolone to cortisol by a human hyperplastic gland at a higher rate than was progesterone when incubated concurrently (Weliky & Engel, 1963), but, although they suggested that progesterone served as a precursor primarily for the formation of corticosterone, other <u>in vitro</u> studies with human glands (Mulrow <u>et al</u>., 1962) indicated that, to the contrary, progesterone was the favoured substrate for cortisol formation when compared with 17α-hydroxypregnenolone. Progesterone has been found to inhibit the conversion of pregnenolone to progesterone by acetone powder preparations of bovine adrenal glands (Kowal <u>et al</u>., 1964b) and this may explain the variation in the pathway to cortisol noted by Weliky and Engel (1963).

21-hydroxypregnenolone has been isolated from human urine following the administration of ACTH (Dobriner & Lieberman, 1952) and this substance is known to be formed from pregnenolone by the adrenal of rats and rabbits (Pasqualini _et al_., 1964). This steroid has been found to be converted to deoxycorticosterone by bovine adrenal glands (Berliner _et al_., 1962; Kowal _et al_., 1964a) and to deoxycorticosterone and corticosterone by hyperplastic human glands (Pasqualini _et al_., 1964). Similarly, 17α,21-dihydroxypregnenolone can be metabolised to 11-deoxycortisol and cortisol (Pasqualini _et al_., 1964) and 17α,21,11β-trihydroxy-pregnenolone to cortisol (Kowal _et al_., 1964a). Consequently the action of the Δ_5-3β-hydroxysteroid dehydrogenase-isomerase system responsible for the formation of the Δ_4-3ketosteroids can be delayed until the hydroxylations at positions 17,21 and 11 have been completed in part or whole. Apart from the pathway involving the rate of conversion of 17α-hydroxypregnenolone to 17α-hydroxyprogesterone, the relative importance of these routes compared with those involved in the formation of progesterone remains to be elucidated. However, the original concept of an orderly hydroxylation sequence following the oxidation of pregnenolone would require but slight modification.

The principal adrenal androgens are testosterone, dehydroepiandrosterone (DHA), androstenedione, 11β-hydroxy-androstenedione and adrenosterone. DHA is produced in greater

amounts than the remainder, between 15 and 25 mg. per day (Vande-Wiele & Lieberman, 1960), but most occurs as the sulphate ester in both plasma and urine.

Both acetate and cholesterol serve as precursors of the adrenal androgens. In humans, acetate has been converted to DHA *in vivo* (Ungar & Dorfman, 1953) and to DHA, androstenedione and 11β-hydroxyandrostenedione by the *in vitro* incubation of slices of the human gland (Bloch *et al.*, 1956; Bloch & Benerischke, 1959). The formation of androstenedione and 11β-hydroxyandrostenedione from the C_2 precursor has also been shown in animals (Bligh *et al.*, 1955; Bryson & Sweat, 1962).

Cholesterol has been noted to be an *in vivo* precursor of DHA in a case of adrenal carcinoma (Ungar & Dorfman, 1953) and *in vitro* in several human adrenal adenomas (Burstein & Dorfman, 1962; Gual *et al.*, 1962; Goldstein *et al.*, 1963), and in the foetal adrenal gland (Villee *et al.*, 1959).

11β-hydroxyandrostenedione has been extracted from normal human adrenal glands (Neher, 1958) while DHA and testosterone have been found in adrenal tumours (Anliker *et al.*, 1956; Plantin *et al.*, 1957) and androstenedione and adrenosterone in animal glands (Reichstein, 1936; Reichstein & Ewu, 1941). Only 11β-hydroxyandrostenedione has been found to be formed *in vitro* from endogenous substrates (Cooper *et al.*, 1955; Cooper *et al.*, 1958;

Dyrenfurth *et al.*, 1960).

In 1953, Lieberman and Teich suggested that the conversion of pregnenolone to 17α-hydroxypregnenolone might represent the first step in the formation of androgens. Both steroids are good substrates for the formation of DHA, androstenedione and 11β-hydroxyandrostenedione *in vitro* by normal and pathological human adrenal glands (Goldstein *et al.*, 1960; Solomon *et al.*, 1960; Roberts *et al.*, 1961; Bernstein & Dorfman, 1962; Gual *et al.*, 1962; Weliky & Engel, 1962; Cohn *et al.*, 1963; Weliky & Engel, 1963), but only in the testis of the human and of the rat has 17α-hydroxypregnenolone been found to be converted to testosterone (Carstensen, 1961). The extra-adrenal formation of DHA from both pregnenolone and 17α-hydroxypregnenolone has been suggested as a function of striated muscle (Oertel & Eik-Nes, 1959). Both DHA and androstenedione have been produced on incubation of human adrenal tissue with 17α,21-dihydroxypregnenolone (Pasqualini *et al.*, 1964).

Although pregnenolone appears to be an obligatory intermediate in the synthesis of cortisol and corticosterone, its formation is not a prerequisite for the production of the C_{19} adrenal androgens. The formation of 17α,20α-dihydroxycholesterol as the only intermediate between cholesterol and DHA has been reported (Bernstein & Dorfman, 1962; Gual *et al.*, 1962).

Cholesterol may also be bypassed in the formation of androgensby the hog adrenal (Bligh _et al._, 1955), but recently another pathway from cholesterol to DHA has been discovered. DHA as the sulphate ester, has been found to be formed by homogenates of human normal adrenal glands and tumours (Adams, 1963; Cohn _et al._, 1963; Migeon, 1963; Wallace & Lieberman, 1963; Adams, 1964; Boström _et al._, 1964; Killinger & Solomon, 1965) and DHA-sulphate has been isolated from the adrenal venous blood of a patient harbouring an adrenal tumour (Baulieu, 1962). A recent study by Calvin and his colleagues (1963) demonstrated that pregnenolone sulphate - ^{35}S could be metabolised _in vivo_ to ^{35}S labelled DHA-sulphate. The conversion of pregnenolone sulphate to 17α-hydroxypregnenolone sulphate has been reported in an incubation of hyperplastic adrenal tissue (Calvin & Lieberman, 1964), but although pregnenolone was converted to pregnenolone sulphate, 17α-hydroxypregnenolone sulphate and DHA-sulphate by an homogenate of a normal human adrenal gland, pregnenolone sulphate was not metabolised under these _in vitro_ conditions (Killinger & Solomon, 1965). The demonstration of the presence of cholesterol sulphate in beef adrenal glands (Drayer _et al._, 1964) and the _in vivo_ conversion of cholesterol-7α-^{3}H-sulphate-^{35}S to DHA containing both isotopes in a case of adrenal carcinoma has revealed the presence of another biosynthetic route for the formation of androgens (Roberts _et al._, 1964). As

DHA-sulphate and DHA are freely interconvertible (Roberts *et al.*, 1961), the supply of biologically active androgen may be regulated by such a mechanism. However, as DHA-sulphate can also be converted to oestrogens (Siiteri & MacDonald, 1963; Baulieu & Dray, 1963), this metabolic pathway may provide not only a reserve pool of androgen, but also be part of a general biosynthetic pathway.

Adrenal androgens, with the exception of DHA, may also be formed by a route involving progesterone. This steroid can be converted to 17α-hydroxyprogesterone and subsequent side chain cleavage by a desmolase to yield androstenedione as was first demonstrated in the rat by Savard and his colleagues (1956) and Slaunwhite and Samuels (1956). This pathway has been shown to occur in other animals (Rao & Heard, 1957: Kushinsky, 1963) and in human adrenal tissue, normal, hyperplastic and neoplastic (Solomon *et al.*, 1958; Kase and Kowal, 1962; Villee *et al.*, 1962; Weliky & Engel, 1962; Roversi *et al.*, 1963; Ward & Grant, 1963).

By a double isotope technique, Kase and Kowal (1962) demonstrated that the yield of androstenedione and testosterone from the incubation of a normal human adrenal gland was greater from progesterone than 17α-hydroxyprogesterone. This accords with the finding of a biosynthetic pathway from progesterone to testosterone with testosterone acetate as the intermediate in *Cladosporium resinae* (Fonken *et al.*, 1960) and in human testes

and ovaries (Dorfman, 1962), so that the formation of 17α-hydroxyprogesterone from progesteron may not be an obligatory step. Testosterone may also be formed from DHA *via* Δ_5-androstenediol, thereby omitting androstenedione as an intermediate (Baulieu *et al.*, 1963b).

DHA itself may serve as an intermediate in the *in vitro* (Meyer *et al.*, 1955; Rosenfeld *et al.*, 1955; Rubin *et al.*, 1961; Bloch *et al.*, 1962; Rubin *et al.*, 1963) and *in vivo* (Mahesh & Greenblatt, 1962; Dorfman *et al.*, 1963) formation of the Δ_4-3-ketosteroid C_{19} androgens.

Consequently, seven different pathways for the formation of adrenal androgens exist. Most recent evidence tends to point to cleavage of the C_{21} side chain of the Δ_5-3β-hydroxy-pregnenes as being the most important method of their formation with the subsequent action of the Δ_5-3β-hydroxysteroid dehydrogenase systems upon DHA (Baulieu *et al.*, 1963a; Cohn & Mulrow, 1963).

CORTICOSTEROIDS AND ANDROGENS PRESENT IN ADRENAL VENOUS BLOOD

The biosynthetic schemes of the adrenal cortex have mainly been established by the study of *in vitro* one step transformations. By using radioactive substrates, many products have been detected, but such experiments give no indication as to which steroids represent the physiological secretions. This depends upon the

demonstration that a given steroid appears in the adrenal venous blood or plasma in a concentration higher than that found in peripheral blood or plasma.

Cortisol and corticosterone are quantitatively the most important C_{21} steroids present in the human adrenal effluent, in which they have been detected in patients with normal adrenal function (Romanoff et al., 1953; Hudson & Lombardo, 1955; Sweat, 195 Grant et al., 1957; Lombardo et al., 1959; Short, 1960) with either virilism (Bush et al., 1956) or Cushing's syndrome (Sweat, 1955). Cortisol is always the major component of normal adrenal function and is present usually in ten times the concentration of corticosterone although the ratio is variable (Pincus & Romanoff, 1953). In vivo cortisol and corticosterone secretion rates confirm these findings (Petersen & Wyngaarden, 1955; Ayres et al., 1957). Although the level of both cortisol and corticosterone is elevated in Cushing's syndrome, only the level of cortisol rises wit the administration of exogenous ACTH (Hudson & Lombardo, 1955; Grant et al., 1957).

Progesterone, 17α-hydroxyprogesterone, 11-deoxycortisol and cortisone also have been detected in normal adrenal venous blood but always in trace amounts (Lombardo et al., 1959; Touchstone et al., 1959; Short, 1960), so that they have not been found in every sample assayed (Lombardo et al., 1959). However, after

the administration of ACTH, the amounts of these substances are increased in the adrenal blood (Short, 1960).

11β-hydroxyandrostenedione has been detected in adrenal venous plasma of normal (Romanoff et al., 1953; Sweat, 1955; Grant, et al., 1957; Lombardo et al., 1959; Short, 1960) and virilised patients (Bush et al., 1956), so that it was thought to be the major secreted androgen (Bush & Mahesh, 1959). Trace amount of androstenedione have also been isolated (Sweat, 1955; Short, 1960). However, testosterone has only recently been demonstrated in the peripheral blood and shown to be elevated due to adrenal adenomas (Dorfman et al., 1963).

The magnitude of the amount of DHA present in normal urine has been appreciated only since 1960 (Vande-Wiele & Lieberman, 1960 Almost all occurs as the 3-sulphate ester (Munson, et al., 1944) and it has been found in both peripheral and adrenal venous blood in this form (Migeon & Plager, 1954; Clayton et al., 1955; Baulieu, 1962). Trace quantities of free DHA do occur in the peripheral (Cohn et al., 1961) and adrenal venous blood of normal and virilised patients (Dorfman & Ungar, 1953; Bush et al., 1956; Bush & Mahesh, 1959; Hirschmann et al., 1960; Short, 1960). The adrenal gland itself secretes DHA-sulphate as mentioned above and as has been shown by in vitro techniques (Cohn et al., 1963; Migeon, 1963; Wallace & Lieberman, 1963; Boström et al., 1964). A further C_{19} steroid, 7-keto-DHA has been isolated from normal

and pathological human urines (Fukushima & Gallagher, 1957) and as 7-keto-DHA-sulphate from the adrenal venous blood in a case of an adrenal tumour (Baulieu, 1962).

ADRENOCORTICAL BIOSYNTHETIC ENZYME SYSTEMS

The most important positions of the steroid molecule which are hydroxylated by the adrenal glands of both man and animals are those at carbon atoms 17,21 and 11 of the steroid nucleus. The 21 and 17α-hydroxylases are located in the soluble fraction of the cell (Plager & Samuels, 1953), while the site of the 11β-hydroxylase activity is in the mitochondria (Sweat, 1951; Hayano & Dorfman, 1953). All three enzyme systems require reduced NADP and the 11β-hydroxylase also uses molecular oxygen (Hayano *et al*., 1956). The original hypothesis was that the hydroxylations of the progesterone molecule occurred in positions 17, 21 and 11 in that order (Hechter & Pincus, 1954; Eichhorn & Hechter, 1957). Once 21-hydroxylation has occurred, an effective block to 17α-hydroxylation became apparent. Similarly, when an oxygen function was substituted at the 11β-position, no further hydroxylations occurred. However, the conversion of 11β-hydroxyprogesterone to further hydroxylated steroids by tissue of the zona glomerulosa of the human adrenal cortex has been observed (Grant, 1962). The possibility that more than one 11β-hydroxylase

exists has been raised (Brode, 1962; Sweat, 1962) and recent evidence obtained from a study of the urinary products, found in cases of congenital adrenal hyperplasia, postulated the presence of different 21-hydroxylase systems (Degenhert _et al._, 1965). Only one 17α-hydroxylase appears to be present in human adrenal tissue.

Hydroxylations may occur at other positions of the steroid structure. With the exception of those occurring at positions 16 and 19, no biological significance can as yet be attached to their presence.

Two mitochondrial enzyme systems capable of causing hydroxylation in either the 6α or 6β positions have been reported (Meyer _et al._, 1955). Both 6α-hydroxyandrostenedione and 6α,11β-dihydroxyandrostenedione have been found in adrenal tissues and a 6β-hydroxy group has been shown to be added to progesterone, 17α-hydroxyprogesterone, androstenedione, deoxycorticosterone, corticosterone and cortisol (Hechter _et al._, 1951; Haines, 1952; Hayano & Dorfman, 1952; Meyer _et al._, 1955; Neher & Wettstein, 1956; Nowaczynski _et al._, 1962; Goldstein _et al._, 1963; Pasqualini _et al._, 1964).

16α-hydroxylation can occur in both animal (Rao & Heard, 1957; Wettstein _et al._, 1959) and human adrenal glands (Villee _et al._, 1962; Weliky & Engel, 1963). In one instance,

16α-hydroxyprogesterone was the major product from the incubation of a hyperplastic human adrenal gland with progesterone-4-^{14}C as substrate (Weliky & Engel, 1963) 5-pregnene-3α,16α,20α-triol has been isolated from the urine of a patient who had an adrenal carcinoma (Fukushima et al., 1961).

Incubation and perfusion techniques have shown the presence of a 19-hydroxylating enzyme system, located in the mitochondria and shown to affect androstenedione, deoxycorticosterone and 11-deoxycortisol (Hayano & Dorfman, 1955; Kahnt et al., 1955; Levy & Kushinsky, 1955; Meyer, 1955a).

The importance of this enzyme system may well be as an intermediate step in the formation of adrenal oestrogens in that incubation of adrenal tissue with 19-hydroxyandrostenedione yielded oestrone (Meyer, 1955b), while that of the 16α-hydroxylase system may be involved in the conversion of oestrone to oestriol by the adrenal (Engel, 1962).

The presence of an adrenal enzyme system capable of converting both C_{21} and C_{19} Δ_5-3β-hydroxysteroids to those with a Δ_4-3-ketone grouping was demonstrated by Samuels and his associates (1951). This Δ_5-3β-hydroxysteroid dehydrogenase system (Δ_5-3βHSD) was shown to required NAD (Samuels et al., 1951), to be a lipoprotein, and to be located in all cell fractions, but appeared in highest concentrations in the microsomes

(Beyer & Samuels, 1956). It was thought that this system effected both the oxidation ofthe β hydroxyl group at position 3 and the shift in the double bond from the 5-6 to the 4-5 position. Working with the micro-orgamism Pseudomonas testosteroni, Talalay and Wang (1955) showed the presence of a separate isomerase component, effecting only the change in the position of the double bond from ring B to ring A. A direct transfer of a proton from position 4 to 6 on the enzyme surface without exchange with the media was also demonstrated. Isomerase activity could be shown to be present in rat liver and human peripheral plasma.

Beyer and Samuels (1956) used pregnenolone as the substrate for this enzyme system, measuring its activity by the isolation of the formed progesterone. However, the Δ_5-3β-hydroxy 17-ketosteroid, DHA, was subsequently found to be utilized at a higher rate (Rubin & Dorfman, 1957; Kowal et al., 1964a).

This enzyme system is important physiologically as its action is necessary for the formation of the C_{21} and C_{19} Δ_4-3-ketosteroids. In 1961, an impetus to further research into itsfunction was received by the finding of a deficiency of the enzyme in a small number of cases of congenital adrenal hyperplasia associated with virilism and a marked tendency to salt loss (Bongiovani, 1961). A deficiency of the 21-hydroxylase or

11β-hydroxylase occur more commonly in this disorder, but of the aetiology of virilism due to adrenocortical tumours, little or nothing is known. A deficiency of the Δ_5-3βHSD has been reported in such tumours histochemically in tissue sections (Roversi _et al._, 1963; Goldman _et al._, 1964), by the incubation of tissue (Rubin _et al._, 1963; Goldman _et al._, 1964) and by urinary studies (Lipsett & Wilson, 1962). Other enzyme abnormalities have been noted in adrenal tumours by the study of the urinary steroids or their _in vitro_ activity. Deficiencies of the 21 and 11β-hydroxylases have been recorded in some tumours (Lipsett & Wilson, 1962) and an increased activityof the 17α-hydroxylase of the tumour with subsequent desmolase action in another (Roversi _et al._, 1963).

Initially, only one Δ_5-3βHSD system was thought to exist acting upon both C_{21} and C_{19} Δ_5-3β-hydroxysteroids. In an incubation of an hyperplastic adrenal gland, Weliky and Engel (1962), using a double isotope technique, noted the absence of the Δ_5-3βHSD for pregnenolone, yet they isolated cortisol from the medium. They proposed that multiple substrate-specific Δ_5-3βHSD systems may exist in the gland. This was substantiated by the reports that not only did pregnenolone and DHA serve as substrates, but also 17α-hydroxypregnenolone, 21-hydroxypregnenolon 17α,21-dihydroxypregnenolone, 11β,17α,21-trihydroxypregnenolone

and Δ_5-androstenediol (Berliner et al., 1962; Baulieu et al., 1963b; Kowal et al., 1964a; Pasqualini et al., 1964).

Biochemical evidence has now accrued confirming the existence of more than one Δ_5-3βHSD system. This has been shown histochemically in the mouse testis also (Baillie & Griffiths, 1964).

Mammalian isomerase activity appeared to differ from that found in bacterial preparations (Werbin & Chaikoff, 1963) and two substrate specific Δ_5-3-ketosteroid isomerase, each distinguishable from the Δ_5-3β-hydroxysteroid dehydrogenase, were shown to exist in bovine adrenals (Ewald et al., 1964a). The enzymes were named Δ_5-pregnene-3,20-dione isomerase and Δ_5-androstene-3,20-dione isomerase according to the appropriate substrate utilized. Both isomerases were found to be associated with the microsomal and mitochondrial fractions of the cells (Ewald et al., 1964b; Krüskemper et al., 1964), but the Δ_5-pregnene-3,20-dione isomerase was more tightly bound to the cell components then the Δ_5-androstene-3,20-dione isomerase. Neither isomerase showed any coenzyme requirements (Krüskemper et al., 1964).

In a further investigation of the Δ_5-3βHSD system in bovine adrenal glands, Kowal and his colleagues (1964b), using acetone powder preparations, noted that both DHA and more especially androstenedione, inhibited the conversion of pregnenolone by

inhibiting the Δ_5-pregnene-3,20-dione isomerase. This same effect was noted with all the C_{21} Δ_5-3β-hydroxysteroids tested, so that one system appeared to exist capable of acting upon all pregnene derivatives. Progesterone itself also inhibited the conversion of pregnenolone but did so by acting upon the dehydrogenase component of the Δ_5-3βHSD system and not the isomerase. No inhibition of the conversion of DHA to androstenedione was achieved with any C_{21} Δ_5-3β-hydroxysteroid or C_{21} Δ_4-3-ketosteroid tested, thus illustrating the presence of a separate system for DHA. These authors have postulated that the inhibitory effect of DHA, androstenedione, 11β-hydroxyandrostenedione and testosterone upon the Δ_5-3βHSD systems for the pregnenes may explain the low or normal cortisol production in patients possessing virilising adrenal tumours. However, this does not explain the occurrence of Cushing's syndrome and virilism due to adrenal tumours.

The inhibitory effect of progesterone upon the conversion of pregnenolone to progesterone was progressively decreased by subsequent hydroxylation at positions 17, 21 and 11. Consequently, there may be an in-built self-regulatory mechanism to control the concentration of cortisol produced by the adrenal cortex.

Only one dehydrogenase system has been found so far for the Δ_5-3β-hydroxysteroids, but a separate dehydrogenase does exist

for the saturated 3β-ol compounds (Kowal _et al_., 1964b).

The conversion of a Δ_5-3-alcohol to the corresponding Δ_4-3-ketone by bovine adrenal homogenates never goes to completion (Samuels, 1953). This may be due to the reversibility of the Δ_5-3βHSD system as was demonstrated using acetone powder preparations of sheep adrenal microsomes (Ward & Engel, 1964).

In 1961, when the present work was commenced, little was known about the Δ_5-3βHSD system in the human adrenal cortex. Using histochemical techniques, its distribution had been outlined to be predominantly in the clear cells of the outer zona fasciculata (Wattenberg, 1958; Dawson _et al_., 1961).

A technique was evolved by Rubin and her colleagues (1961) which allowed measurement of the activity of the Δ_5-3βHSD system by the _in vitro_ incubation of adrenal tissue using DHA as substrate and determination of the amount of androstenedione formed. The system was shown to be present in the adrenal glands of both man and animals.

Consequently, it appeared of interest to study in more detail the occurrence of the Δ_5-3βHSD system in normal and pathological human adrenal tissues and to see if the administration of ACTH or cortisol or one of its synthetic analogues affected the activity of this enzyme system.

MATERIAL AND METHODS

The following trivial names have been used in the text.

Trivial Name	Substance
ACTH	adrenocorticotrophic hormone
adrenosterone	Δ_4-androstene-3,11,17-trione
aldosterone	Δ_4-pregnene-11β,21-diol-18-al-3,20-dione
Δ_5-androstenediol	Δ_5-androstene-3β,17β-diol
androstenedione	Δ_4-androstene-3,17-dione
cholesterol	Δ_5-cholestene-3β-ol
corticosterone	Δ_4-pregnene-11β,21-diol-3,20-dione
cortisol	Δ_4-pregnene-11β,17α,21-triol-3,20-dione
cortisone	Δ_4-pregnene-17α,21-diol-3,11,20-trione
dehydroepiandrosterone (DHA)	Δ_5-androstene-3β-ol-17-one
deoxycorticosterone (DOC)	Δ_4-pregnene-21-ol-3,20-dione
11-dehydrocorticosterone	Δ_4-pregnene-21-ol-3,11,20-trione
11-deoxycortisol	Δ_4-pregnene-17α,21-diol-3,20-dione
11-deoxy,19-hydroxycortisol	Δ_4-pregnene-17α,19,21-triol-3,20-dione
6α-11β-dihydroxyandrostenedione	Δ_4-androstene-6α,11β-diol-3,17-dione

Trivial Name	Substance
17α,20α-dihydroxycholesterol	Δ_5-cholestene-3β,17α,20α-triol
17α,21-dihydroxypregnenolone	Δ_5-pregnene-3β,17α,21-triol-20-one
6β,17α-dihydroxyprogesterone	Δ_4-pregnene-6β,17α-diol-3,20-dione
6α-hydroxyandrostenedione	Δ_4-androstene-6α-ol-3,17-dione
7α-hydroxyandrostenedione	Δ_4-androstene-7α-ol-3,17-dione
7α-hydroxy-DHA	Δ_5-androstene-3β,7α-diol-17-one
7α-hydroxy-11-deoxycortisol	Δ_4-pregnene-7α,17α,21-triol-3,20-dione
7α-hydroxyoestrone	$\Delta_{1,3,5(10)}$-estration-3β,7α-diol-17one
7α-hydroxypregnenolone	Δ_5-pregnene-3β-7α-diol-20-one
16α-hydroxyprogesterone	Δ_4-pregnene-16α-ol-3,20-dione
17α-hydroxypregnenolone	Δ_5-pregnene-3β,17α-diol-20-one
17α-hydroxyprogesterone	Δ_4-pregnene-17α-ol-3,20-dione
20α-hydroxycholesterol	Δ_5-cholstene-3β,20α-diol
6β-hydroxyandrostenedione	Δ_4-androstene-6β-ol-3,17-dione
6β-hydroxycorticosterone	Δ_4-pregnene-6β,11β,21-triol-3,20-dione
6β-hydroxycortisol	Δ_4-pregnene-6β,11β,17α,21-tetrol-3,20-dione
6β-hydroxydeoxycorticosterone	Δ_4-pregnene-6β,21-diol-3,20-dione
6β-hydroxyprogesterone	Δ_4-pregnene-6β-ol-3,20-dione

Trivial Name	Substance
7β-hydroxy-DHA	Δ_5-androstene-3β,7β-diol-17-one
7β-hydroxypregnenolone	Δ_5-pregnene-3β,7β-diol-20-one
11β-hydroxyandrostenedione	Δ_4-androstene-11β-ol-3,17-dione
19-hydroxyandrostenedione	Δ_4-androstene-19-ol-3,17-dione
19-hydroxydeoxycorticosterone	Δ_4-pregnene-19,21-diol-3,20-dione
21-hydroxypregnenolone	Δ_5-pregnene-3β,21-diol-20-one
7-keto-DHA	Δ_5-androstene-3β-ol-7,17-dione
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
pregnenolone	Δ_5-pregnene-3β-ol-20-one
progesterone	Δ_4-pregnene-3,20-dione
testosterone	Δ_4-androstene-17β-ol-3-one
17α,21,11β-trihydroxy-pregnenolone	Δ_5-pregnene-3β,11β,17α,21-tetrol-20-one

TISSUE PREPARATION

Human adrenal glands were removed surgically from patients with metastatic mammary carcinoma or various adrenal disorders, and placed in polythene containers on ice for transit to the laboratory.

Bovine glands were obtained from Walter Black and Company

Limited, Glasgow. Mouse adrenal glands were obtained from the Animal House of the Glasgow Royal Infirmary Pathology Department.

INCUBATION PROCEDURES

The adrenal glands were freed of connective tissue and fat, weighed, chopped and minced in a Latapie Mill. The resultant pulp wa homogenized in a hand piston-type glass homogeniser with a small volume of the appropriate medium, and diluted to a final concentration (W/V) of 100 mg./ml., 200 mg/ml. or 300 mg/ml.

The homogenates were incubated in a Gallenkamp metabolic shaker at 37°C in air, or in an atmostphere of 95% oxygen and 5% carbon dioxide when Krebs' Ringer bicarbonate medium was used.

MEDIA

Three different media were employed.

(a) Equal volumes of 0.154M sodium chloride and 0.1M phosphate buffer, pH 7.4, the latter being prepared from monopotassium and disodium phosphates.

(b) Krebs' Ringer phosphate, pH 7.4

(c) Krebs' Ringer bicarbonate, pH 7.4

Glucose and nicotinamide were added to both the Krebs' Ringer phosphate and bicarbonate media to a 0.01M final concentration.

SUBSTRATES

Dehydroepiandrosterone (DHA) (L. Light & Co.) was dissolved in propylene glycol to give a concentration of either 20 mg/ml. or 50 mg/ml.

Dehydroepiandrosterone-4-^{14}C (DHA-4-^{14}C) (New England Nuclear Corporation, 141.0 µc/mg; Amersham, 117.0 µc/mg.) was diluted to between 0.45 µc/mg. and 4.84 µc/mg. with unlabelled DHA.

Androstenedione-4-^{14}C (Amersham, 121.7 µc/mg.) was diluted with unlabelled androstenedione (L. Light & Co.) to give a final specific activity of 3.44 µc/mg.

Pregnenolone-16T (Amersham, 4.97 mc/mg.) was diluted with unlabelled pregnenolone (L. Light & Co.) to 0.5 mc/mg.

17α-hydroxypregnenolone-7T (New England Nuclear Corporation; 44.3 mc/mg.) was diluted with unlabelled 17α-hydroxypregnenolone to give a final specific activity of 21.3 µc/mg.

Paper chromatography of these four substrates revealed only one zone of radioactivity.

Progesterone-4-^{14}C (Amersham, 83.0 µc/mg.) was used with the supplied specific activity. Paper chromatography was not carried out to ensure a single radioactive zone.

Cholesterol-4-^{14}C (Amersham 50.0 µc/mg.) was diluted with authentic cholesterol (L. Light & Co.) to give a specific activity of 4.81 µc/mg.

COFACTORS

Diphosphopyridine nucleotide (NAD) (L. Light & Co.) was dissolved in 0.154M sodium chloride to give a concentration of 30 mg./ml.

SOLVENTS

All solvents were of BDH "Analar" quality and were redistilled before use.

EXTRACTION PROCEDURES

All the incubates were extracted thrice with 5 volumes of either ethyl acetate for low concentrations of tissue, or three times with 5 volumes of benzene:chloroform (6:1) with large amounts of tissue. The pooled extracts were evaporated to dryness under a stream of air at 45°C.

COLUMN CHROMATOGRAPHY

Silica Gel Davison (USA) Grade 12 (L. Light & Co.) was used without further activation.

Aluminum oxide, BDH "for chromatography" grade was employed, also without prior activation.

PAPER CHROMATOGRAPHY

Whatman No. 1 paper for chromatography was used throughout. The paper was washed in a soxhlet extractor with chloroform, benzene, ethyl acetate and methanol prior to use.

The paper chromatographic systems used were those of Zaffaroni (Zaffaroni & Burton, 1951; Burton et al., 1951), Savard (1953) and Bush (1952).

SPECTROPHOTOMETRY

A Unicam SP500 spectrophotometer was used to determine the absorption of light at 225 mμ, 240 mμ and 255 mμ by steroids absorbi[ng] in the ultra-violet. The criterion of a reliable curve was the finding of an optical density at both 225 mμ and 255 mμ which was approximately half the optical density at 240 mμ. Steroids possessing a 17-ketone group with or without a Δ_4-3-ketone structure, were determined by the Zimmermann reaction (Zimmermann 1935; Fotherby, personal communication).

DETECTION AND QUANTITATION OF RADIOACTIVE STEROIDS

Radioactive steroids were located on paper chromatograms using a windowless gas-flow Nuclear Chicago 4π Actigraph II.

Samples for quantitation were plated at "infinite thinness" and counted by an Isotope Development Limited (IDL) end-window

automatic solid sample counting 6014 system, operating at 6% efficiency for carbon-14 (1.32×10^5 cpm/µc-^{14}C). A Tracer-Lab SC16, windowless gas-flow solid sample counting system connected to a Panax Scaler (T300) was used for tritium, operating at 32% efficiency (7.04×10^5 cpm/µc - tritium). Constancy of specific activity of the isolated steroids was considered established if the variation in the specific activity of the steroid and any derivatives made was within the 5% standard error of net activity (Calvin et al., 1949).

FORMATION OF STEROID DERIVATIVES

Acetylation.

Acetylation was performed by dissolving the steroid in pyridine and adding an equal volume of acetic anhydride, and leaving overnight at room temperature (DeCourcy et al., 1953).

Saponification.

The steroid acetates were hydrolysed using 0.4% methanolic potassium bicarbonate according to the method of Meyer (1953). This method was found to be unsuitable for the complete hydrolysis of 3β-acetoxyl groups for which a method using methanolic sodium hydroxide was substituted (Bush, 1961).

Oxidation.

The oxidation of certain side chains to give 17-ketones was

performed using acetic acid and a 2% (W/V) aqueous solution of chromium trioxide according to the method of Lieberman and his colleagues (1953).

ASSAY OF THE Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE SYSTEM UTILIZING DHA

METHODS

The assay procedure is a modification of that proposed by Rubin and her associates (1961). 1.6 ml. of a 20% (W/V) homogenate prepared in equal volumes of 0.154M sodium chloride and 0.1M phosphate buffer (pH 7.4), were added to each flask containing 6 mg. NAD and 500 μg. DHA for a final volume of 1.80 ml. A tissue blank with a similar amount of tissue and equal NAD concentration was also prepared. In all the experiments, at least duplicate incubations were undertaken. Following incubation in air at 37°C for 30 minutes, the media were diluted with 3 volumes of distilled water and extracted three times with 5 volumes of ethyl acetate. The pooled extracts were taken to dryness.

Attempts to determine the concentration of Δ_4-3-ketosteroids formed by direct ultra-violet spectrophotometry of the extracts against the tissue blank extract, as recommended by Rubin and her colleagues (1961) were unsatisfactory. Therefore, the method was modified to include a "preparatory" silica gel column.

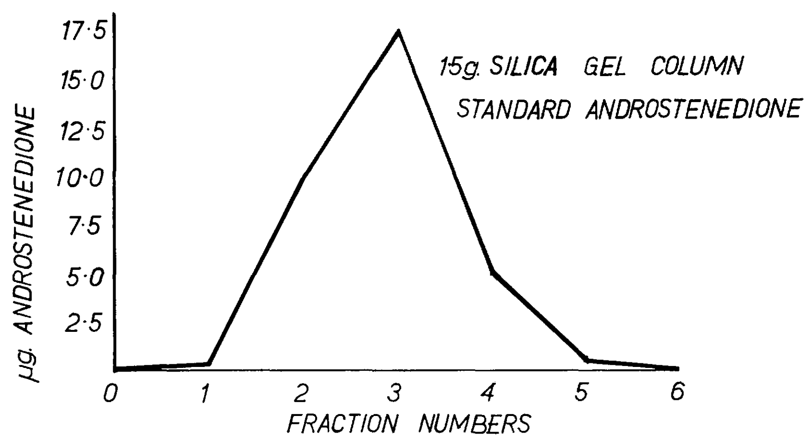
1.5 g. silica gel columns, slurried in benzene, were set up. The extracts were added to the column with two 10 ml. washes of benzene and the steroids were eluted by stepwise increments of ethyl acetate:benzene as outlined in Table XX and Figure 37.

TABLE XX

SILICA GEL COLUMN CHROMATOGRAPHY

Standard Protocol

Fraction number	% Ethyl acetate in benzene	ml. collected
1	100% benzene	30
2	5%	20
3	10%	20
4	20%	20
5	50%	20
6	100% ethyl acetate	20



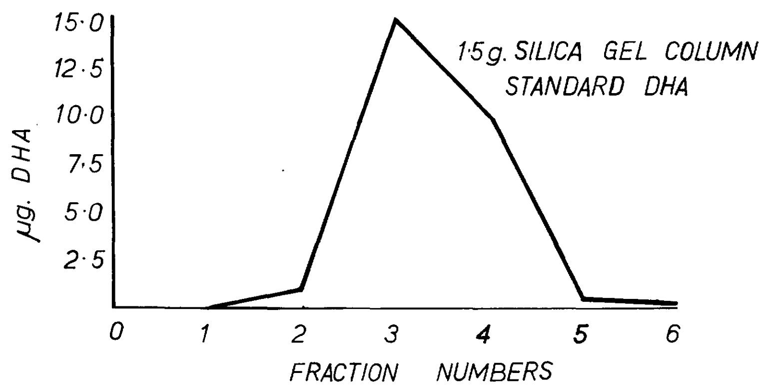


Fig. 37.- Silica gel column chromatography.

No separation of DHA from androstenedione was achieved, but a satisfactory measurement of Δ_4-3-ketosteroids could always be obtained when read against the tissue blank. The recovery of standard DHA and androstenedione from the columns averaged 90%. The recovery of androstenedione, added to a suitable tissue blank and processed in the same manner as the incubation samples, averaged 78%. No correction of the results is made for the losses incurred during extraction and chromatography.

The results are expressed as µg. U.V. absorbing steroid formed per minute per mg. nitrogen. The nitrogen content of the homogenate was determined by the method of Nayyar and Glick (1954).

RESULTS

Seven human adrenal glands, removed from patients with metastatic mammary carcinoma with normal adrenocortical function, were assayed (Table XXI). The results show a wide scatter from 1.36 to 2.50 µg. steroid formed per minute per mg. nitrogen. The assay of eight hyperplastic glands associated with Cushing's syndrome also reveals a broad spectrum of results (Table XXII). The mean rate of production by normal glands is 1.83 ± 0.46 as opposed to 1.94 ± 0.41 µg. per minute per mg. nitrogen for the glands found in Cushing's syndrome (Table XXIII). The statistical difference between the two means is not significant ($P = <0.7 > 0.6$).

TABLE XXI

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Normal Human Adrenal Glands

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 1.6 ml. of 20% (W/V) homogenate
Additions: NAD 6 mg. Final Volume 1.8 ml.
Substrate: DHA 500 µg.
Incubation Time: 30 minutes Gas-phase:air

Gland number	Gland weight g.	Pre-operative Therpay	µg. UV absorbing steroid formed	mg. nitrogen per ml. of homogenate	µg. UV absorbing steroid formed per minute per mg. nitrogen
1	6.10	ACTH, 40 I.U./day for 6 days	209.8	2.30	1.90
2	3.36	-	143.0	2.0	1.49
3	5.40	ACTH, 40 I.U./day for 4 days	229.0	1.80	2.50
4	3.87	-	333.0	2.80	2.47
5	3.28	-	144.0	2.20	1.36
6	3.60	-	139.0	2.0	1.45
7	2.10	Prednisone 30 mg./day for 10 weeks	208.4	2.60	1.67

TABLE XXII

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Cushing's Syndrome - Bilateral Adrenocortical Hyperplasia

Incubation conditions as in Table XXI

Gland number	Gland weight g.	Pre-operative therapy	µg. UV absorbing steroid formed	mg. nitrogen per ml. of homogenate	µg UV absorbing steroid formed per minute per mg. nitrogen
8	6.50	-	201.0	2.0	2.09
9	6.40	-	160.0	2.25	1.47
10	6.50	-	139.0	2.0	1.45
11	-	-	320.0	2.94	2.24
12	7.0	-	172.0	2.30	1.55
13	8.30	-	312.0	3.25	2.0
14	22.20	ACTH, 40 I.U. twice daily for 2 days	160.0	2.10	1.58
			253.0*	2.66	1.97
			352.0*	3.0	2.43
15	21.0	-	275.0	2.80	2.06
			288.0*	2.38	2.53

* Areas of adenomatous hyperplasia

TABLE XXIII

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Comparison of Normal and Hyperplastic Glands

The data recorded in this Table are derived from Tables XXI and XXII

Type of adrenal gland	Total number of glands	µg UV absorbing steroid formed per minute per mg. nitrogen mean ± SD
Normal	7	1.83 ± 0.46
Hyperplastic	8	1.94 ± 0.41

_t_ test $P < 0.7 > 0.6$

In Cushing's syndrome associated with bilateral adrenocortical hyperplasia, an increase in the plasma concentration of ACTH has been reported (Davies _et al_., 1960). Thus each hyperplastic gland has been exposed to high blood levels of ACTH for varying periods of time. Consequently, ACTH of endogenous origin does not appear to alter the activity of the enzyme system measured _in vitro_ under the experimental conditions used here. Moreover, the administration of ACTH pre-operatively, in pharmacological doses, also fails to elicit any change in the activity in either normal or hyperplastic glands. (Glands 1 and 3, Table XXI and Gland 14, Table XXII).

Prednisone, a synthetic analogue of cortisone, causes no change in the activity (Gland 7, Table XXI).

ALTERATION OF THE ASSAY TECHNIQUE

An ideal assay for such an enzyme system is one which not only gives reproducible results, but also requires a small amount of tissue. With these requirements in view, a further modification of the method of assay of the Δ_5-3βHSD system for DHA was undertaken.

METHODS

0.5 ml. of a 10% (W/V) homogenate, prepared in equal volumes

of 0.154M sodium chloride and 0.1M phosphate buffer (pH 7.4), was added to each flask containing 6 mg. NAD and 200 µg. DHA, giving a final volume of 0.7 ml. At least triplicate incubations were prepared, together with a suitable tissue blank similar in all respects except lacking the addition of the substrate DHA. The incubations were performed for 30 minutes in air at 37°C and following dilution with 4 volumes of distilled water, they were extracted three times with 5 volumes of ethyl acetate. The pooled extracts, following evaporation to dryness, were submitted to silica gel chromatography as previously outlined (p. 128).

The results are expressed as µg. U.V. absorbing steroid formed per minute per mg. nitrogen as before.

RESULTS

Direct comparison of the two methods is outlined in Table XXIV. The activity of the enzyme system, expressed in µg. steroid formed per minute per mg. nitrogen, is increased bya factor of between 2 and 2.5 using 50 mg. of tissue compared with the incubation of 320 mg., in both normal and hyperplastic glands. This is most probably related to the relative increase in the concentration of NAD present in the incubates of smaller tissue amounts, the Δ_5-3βHSD having been shown to be NAD dependent

TABLE XXIV

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Comparison of Results Using Different Tissue and NAD Concentrations

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Substrate: (1) DHA, 500 µg. with 1.6 ml. of 20% (W/V) homogenate
(2) DHA, 200 µg. with 0.5 ml. of 10% (W/V) homogenate
Incubation Time: 30 minutes. Gas-phase: air.

Type of gland	Gland weight g.	µg. UV absorbing steroid formed per minute per mg. nitrogen	
		1.6 ml. 20% (W/V) homogenate NAD 3.3 mg./ml.	0.5 ml. 10% (W/V) homogenate NAD 8.6 mg./ml.
Normal	6.10	1.90	4.10
Hyperplastic	8.38	2.0	4.95
Hyperplastic	7.0	1.55	4.10

(Samuels et al., 1951). No further experiments were made to determine the optimal requirements for NAD in human adrenal glands by the Δ_5-3βHSD system. The concentration of NAD used in the initial experiments was that reported to be optimal under similar conditions to the present (Rubin et al., 1961).

Of the eight glands studied by this method, only two were associated with normal in vivo function. The pre-operative administration of ACTH or prednisone did not appear to alter the activity of the enzyme system (Table XXV).

Five hyperplastic glands associated with Cushing's syndrome (Table XXVI) showed activities ranging from 4.0 to 4.98 µg. per minute per mg. nitrogen with a mean of 4.57 ± 0.40 mg. Although there would appear to be a tendency towards higher activity in Cushing's syndrome due to bilateral hyperplasia, statistical confirmation could not be obtained due to the small sample of normal glands. However, glands 18 and 19 (Table XXVI) fall within the activity level found in normal tissue so that it is possible that there is no real difference. The adenoma causing Cushing's syndrome (Gland 23, Table XXVI) exhibits an enzyme activity in the upper range of that found for hyperplastic glands. This is probably related to their high content of clear cells which show a high Δ_5-3βHSD system activity histochemically (Wattenberg, 1958; Dawson et al., 1961) and by the in vitro

TABLE XXV

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Normal Human Adrenal Glands

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 0.5 ml. of 10% (W/V) homogenate
Additions: NAD, 6 mg. Final Volume: 0.7 ml.
Substrate: DHA, 200 µg.
Incubation Time: 30 minutes. Gas-phase: air.

Gland number	Gland weight g.	Pre-operative therapy	µg. UV absorbing steroid formed	mg. nitrogen per ml. of homogenate	µg. UV absorbing steroid formed per minute per mg. nitrogen
16	6.10	ACTH, 40 I.U./day for 4 days	71.9	1.16	4.10
17	2.27	Prednisone 30 mg./day for 3 months	96.9	1.52	4.25

TABLE XXVI

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Cushing's Syndrome - Bilateral Adrenocortical Hyperplasia and an Adrenocortical Adenoma

Incubation Conditions as in Table XXV

Gland number	Gland weight g.	Pre-operative therapy	µg. UV absorbing steroid formed	mg. nitrogen per ml. of homogenate	µg. UV absorbing steroid formed per minute per mg. nitrogen
18	7.0	-	73.6	1.19	4.10
19	8.30	-	94.0	1.56	4.0
20	8.30	-	148.5	2.0	4.95
21	7.20	-	71.0	1.10	4.30
22	8.48	-	100.8	1.35	4.98
23*	18.30	-	132.0	1.87	4.59
			123.0	1.60	5.01
			138.0	2.0	4.60

* Adrenocortioal adenoma - three separate portions incubated

incubation of clear cells with DHA as substrate (Grant, 1962).

The results are summarised in Table XXVII. The finding that variation in the *in vivo* level of ACTH does not alter the overall activity of the enzyme system when determined by either of the described techniques accords with the reports that neither hypophysectomy in rats (Samuels & Helmreich, 1956) nor cobalt irradiation of guinea pig pituitary glands (Rubin & Dorfman, 1957) altered the activity of the Δ_5-3βHSD system utilizing DHA.

Cortisol or its synthetic analogues was without effect upon the enzyme in animal adrenal glands (Rubin & Dorfman, 1957) and was found to be without effect in the two normal human adrenal glands of the present series.

Although the values obtained from both normal and hyperplastic tissue varied from gland to gland, individual values found for any one homogenised gland, using duplicate or triplicate assays, were within 6%. This variation in activity has been demonstrated by other workers in rat, mouse and beef adrenal glands (Samuels, 1953; Rubin *et al.*, 1961) and even between paired glands from the same animal (Rubin *et al.*, 1961). This variability, consequently, would appear to be inherent in the assay of the enzyme system by these procedures in the various species cited, including man.

The value of 1.83 ± 0.46 µg U.V. absorbing steroid formed per minute per mg. nitrogen is much lower than that reported in

TABLE XXVII

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Comparison of Normal and Hyperplastic Glands

The data recorded in this Table are derived from Tables XXV and XXVI

Type of gland	Total number of glands	µg. UV steroid formed per minute per mg. nitrogen mean ± S.D.
Normal	2	4.18
Hyperplastic	5	4.57 ± 0.40

normal human glands by Rubin (1961) and Bloch (1962) and their colleagues. They found 3.9 and 8.2 µg androstenedione to be formed per minute per mg. nitrogen under similar conditions. In an adenoma associated with Cushing's syndrome, values of 2.2 9.3, 28.0 and 31.6 µg. androstenedione formed per minute per mg. nitrogen were reported for four separate areas of the same tumour. Apart from the broad scatter of these figures, these higher values are difficult to reconcile with either of the present series. However, in these published experiments, the U.V. steroids formed were measured spectrophotometrically against a tissue blank directly after extraction. This procedure was never satisfactory in the present series and never yielded satisfactory U.V. absorption curves.

The use of a preparatory column and extraction procedures did not entail losses greater than 22%. Consequently, the difference between the reported results and those of the present ser may be due to the relatively higher purity of the steroids measured spectrophotometrically following column chromatography.

Four tumours have been assayed for their content of the Δ_5-3βHSD system for DHA (Table XXVIII). Gland 24 was removed at operation from a patient suffering from Cushing's syndrome and virilism ("mixed" Cushing's syndrome) and the enzyme activity was much lower than normal. Under similar conditions, Rubin and

TABLE XXVIII

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Tumours associated with Cushing's Syndrome and the Adrenogenital Syndrome

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 1.6 ml. of 20% (W/V) homogenate
Additions: NAD, 6 mg. Final Volume: 1.8 ml.
Substrate: DHA, 500 µg.
Incubation Time: 30 minutes. Gas-phase: air.

Gland number	Clinical diagnosis	Tumour weight g.	Pathological diagnosis	µg. UV absorbing steroid formed per minute per mg. nitrogen
24	Cushing's syndrome	386	Carcinoma	0.169
25	Adrenogenital syndrome	47	Adenoma	0.90
26	Adrenogenital syndrome	1,500	Carcinoma	Nil
27	Adrenogenital syndrome	228	Adenoma	Nil

her co-workers (1963) studied a carcinoma associated with Cushing's syndrome and also noted that it possessed a lower enzyme activity than normal.

Of the three tumours associated with the adrenogenital syndrome, two (Glands 26 & 27, Table XXVIII) did not possess measurable Δ_5-3βHSD activity. This accords with the reports of an absence of the enzyme system in virilising tumours as was shown on *in vitro* incubation by Goldman and his colleagues (1964) and histochemically by Roversi and his associates (1963).

However, not all virilising tumours exhibit an absolute deficiency of this enzyme system, but can show lower than normal activities (Gland 25, Table XXVIII; Rubin *et al*., 1963; Goldman *et al*., 1964).

The relative deficiency of the Δ_5-3βHSD system demonstrated here in a tumour causing Cushing's syndrome and one causing the adrenogenital syndrome gives no indication as to whether the activity of each cell is low or whether some cells lack the enzyme completely, while others possess it in normal amounts. The histochemical studies reported in the first part of this thesis favour the former hypothesis that all cells possess a lowered enzymic activity; however, Goldman and his associates (1964) demonstrated histochemically that the enzyme was only present in certain focal areas of one tumour, associated with virilism, studied by them.

ASSAY OF THE Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE SYSTEM UTILIZING DHA-4-^{14}C

The assay of the Δ_5-3βHSD system using unlabelled DHA as substrate allows comparison of the activity of this enzyme system between various types of human adrenal glands, but it contributes no information as to the number and relative proportions of steroids formed from DHA. Nor does it ensure that androstenedione the expected product is the only U.V. absorbing steroid formed. Moreover, it is possible that androstenedione may be formed from other endogenous sources which may vary in proportion from gland to gland. Consequently, to ensure that the assay technique gave a valid measure of the activity of the enzyme system, it was decided to substitute DHA-4-^{14}C for the unlabelled DHA, used previously, as the substrate and to estimate the radio-active steroids formed by such incubations and to determine whether all the androstenedione formed was derived only from the added substrate.

METHODS

The standard assay procedure, using 0.5 ml. of a 10% (W/V) homogenate prepared in equal volumes of 0.154 M sodium chloride and 0.1M phosphate buffer with 6.0 mg NAD added, was retained. 200 µg. of DHA-4-^{14}C were added to each flask in 0.01 ml. propylene glycol.

The specific activity of the labelled substrate varied from 60 cpm/µg. to 640 cpm/µg., but was constant within each incubation. The incubates were extracted with 5 volumes of ethyl acetate after a 30 minute incubation as previously described.

Alumina column chromatography was substituted for the silica gel column procedure. 7.5 g. alumina columns were established in 0.225% ethanol in benzene, and the extracts were eluted with stepwise increments of ethanol in benzene, 32 fractions being collected. The standard alumina column protocol is shown in Table XXIX.

From Figure 38a it may be seen that good separation of the authentic standard steroids, androstenedione, DHA and 11β-hydroxyandrostenedione, was achieved, the average recovery of each compound being 90%. However, 11β-hydroxyandrostenedione could not be separated from adrenosterone or testosterone by this column procedure.

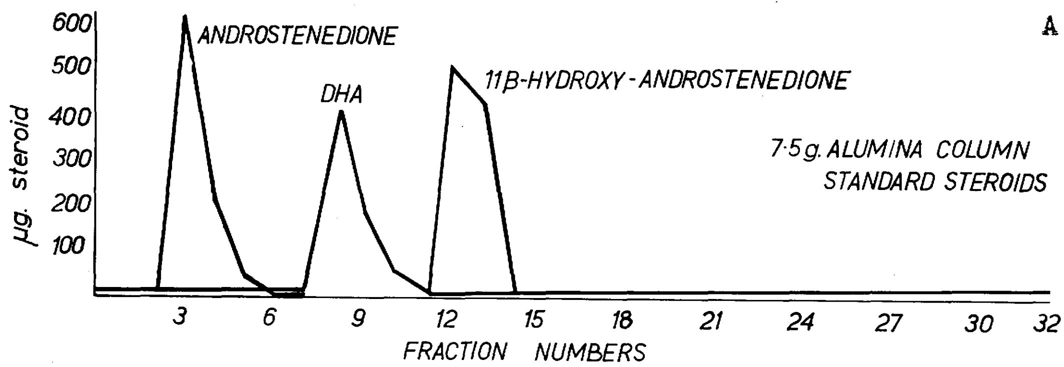
Ultra-violet absorbing steroids were measured spectrophotometrically at 240 mµ and DHA was estimated by the Zimmermann reaction (Zimmermann, 1935; Fotherby, personal communication). Aliquots of each column fraction were assayed for radio-activity and the specific activity of each isolated steroid was determined. Constancy of specific activity was achieved by repeated paper chromatography in several of the systems of Bush (1952) without

TABLE XXIX

ALUMINA COLUMN CHROMATOGRAPHY

Standard Protocol

Fraction number	% ethanol in benzene	ml. collected	Fraction number	% ethanol in benzene	ml. collected
1	0.225	30	17	0.925	20
2	0.250	30	18	0.950	20
3	0.275	30	19	0.975	20
4	0.275	5	20	1.0	20
5	0.300	5	21	2.0	20
6	0.300	30	22	3.0	20
7	0.400	30	23	4.0	20
8	0.500	30	24	5.0	20
9	0.600	20	25	6.0	20
10	0.700	20	26	7.0	20
11	0.750	20	27	8.0	20
12	0.800	20	28	10.0	20
13	0.825	20	29	20.0	20
14	0.850	20	30	40.0	20
15	0.875	20	31	50.0	20
16	0.900	20	32	100.0	30



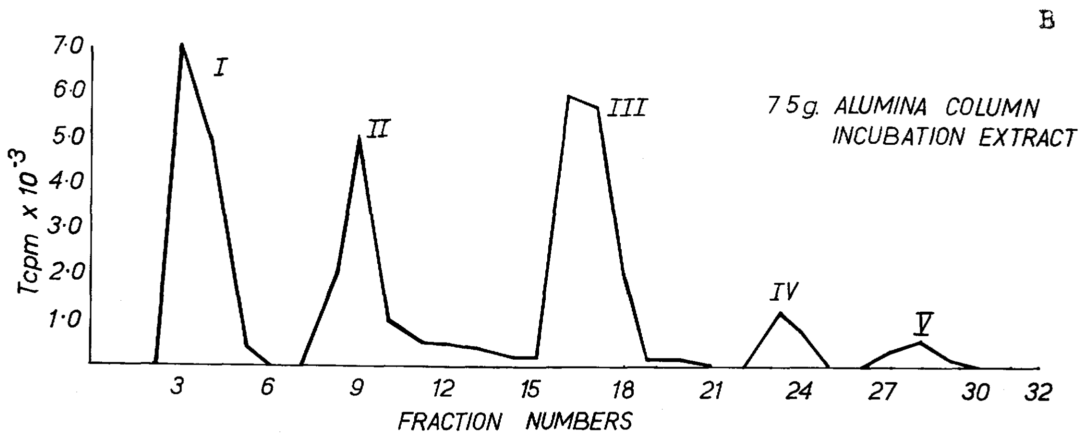


Fig. 38.- Alumina column chromatography.

the addition of carrier steroid. Acetylation of DHA and subsequent hydrolysis were performed in the initial experiments to confirm its identity. Androstenedione was rechromatographed with authentic carrier androstenedione for its identification. Constancy of specific activity from columns and several paper systems was considered to be sufficient identification.

The results are expressed as µg. U.V. absorbing steroid formed per minute per mg. nitrogen of the original homogenate. No correction for extraction and column recoveries is made.

RESULTS

Alumina column chromatography of extracts of the incubates revealed the presence of five radio-active peaks (Fig. 38b). Peaks I and II corresponded to authentic androstenedione and DHA respectively. A prominent shoulder to peak II was observed in a few assays possibly representing trace amounts of 11β-hydroxyandrostenedione, adrenosterone and testosterone, while peaks III, IV and V did not match the mobility of any of the following authentic steroids: 11β-hydroxyandrostenedione, 6β-hydroxyandrostenedione, 19-hydroxyandrostenedione.

The steroid corresponding to peak III absorbed U.V. light and gave a positive Zimmermann reaction with dinitrobenzene. Its identity at this time was unknown, but it was assumed to be

a 17-ketosteroid possessing a Δ_4-3-ketone grouping in Ring A of the steroid molecule. In no instance did the steroid peaks IV and V, eluted from the columns, give U.V. absorption curves. This was assumed to be due to their being either Δ_5-3β-hydroxysteroids or Δ_4-3-ketosteroids present in only trace amounts.

Consequently under the present conditions, the assay of the Δ_5-3βHSD using DHA-4-^{14}C as the substrate revealed two principal products which absorbed U.V. light, one of which was identified as androstenedione and the second of unknown structure.

Two normal adrenal glands, removed surgically from patients with metastatic mammary carcinoma, and five hyperplastic glands removed at operation from patients with Cushing's syndrome, were assayed using this procedure. Tables XXX and XXXI show the µg. of DHA recovered and the µg. of androstenedione and of the unknown steroid (peak III) formed by the incubations of these glands. Each value represents the results of a single assay with the exception of gland 34 in which three separate assays of the same homogenate were examined. In each assay, the specific activity of both isolated steroids lies within 10% of the substrate DHA-4-^{14}C isolated after incubation, this figure being within the 5% standard error of net activity used (Calvin _et al._, 1949; p.126).

TABLE XXX

Δ_5-3βHYDROXYSTEROID DEHYDROGENASE ASSAY

Normal and Hyperplastic Human Adrenal Glands

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 0.5 ml. of 10% (W/V) homogenate
Additions: NAD, 6.0 mg. Final Volume 0.7 ml.
Substrate: DHA-4-^{14}C, 200 μg., specific activity varying from 60 cpm/μg. to 640 cpm/μg.
Incubation Time: 30 minutes. Gas-phase: air.

Gland number	Type of gland	Gland weight g.	Isolated Steroids			
			DHA (peak II)*		Androstenedione (peak I)*	
			μg.	Specific activity ≠ cpm/μg.	μg.	Specific activity ≠ cpm/μg.
28	Normal	3.84	122	71	34	72
29	Normal	3.50	95	60	21	61
30	Hyperplasia	8.30	61	640	71	662
31	Hyperplasia	7.78	98	216	36	214
32	Hyperplasia	8.30	101	234	37	233
33	Hyperplasia	8.48	92	480	66	468
34	Adenomatous hyperplasia	10.0	76	167	59	155
			90	107	30	105
			80	162	30	153

* see Fig. 38b.
≠ counts per minute

TABLE XXXI

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Normal and Hyperplastic Human Adrenal Glands

Incubation conditions as in Table XXX

Gland number	Type of gland	Gland weight g.	Isolated Steroids			
			DHA (peak I)*		Peak III*	
			µg.	Specific activity cpm/µg. ≠	µg.	Sepcific activity cpm/µg. ≠
28	Normal	3.84	122	71	27	73
29	Normal	3.50	95	60	53	60
30	Hyperplasia	8.30	61	640	54	620
31	Hyperplasia	7.78	98	216	39	210
32	Hyperplasia	8.30	101	234	46	243
33	Hyperplasia	8.48	92	480	18	435
34	Adenomatous hyperplasia	10.0	76	167	38	186
			90	107	59	107
			80	162	65	154

* see Fig. 38b.
≠ counts per minute

Thus both steroids are derived only from DHA under these conditions. The sums of the Δ_4-3-ketosteroids formed from the substrate are expressed as µg U.V. absorbing steroids formed per minute per mg. nitrogen and are shown in Table XXXII.

The mean rate of production $\pm$ SD of U.V. absorbing steroids in hyperplastic glands is 4.79 ± 0.51 per minute per mg. nitrogen. Considerable variation in the activity of this enzyme system is apparent between individual hyperplastic glands. This may be related to variable amounts of compact cell tissue from the zona reticularis, which givés anegative histochemical reaction for the Δ_5-3βHSD, included in the homogenates from individual glands. An explanation of the varying amounts of formed androstenedione and the unknown steroid found in the same homogenate from gland 34 is difficult to explain. Although androstenedione composed between 32% and 61% of the total steroid formed by the incubation of this gland, less than 5% difference was detected between these three homogenates in their production of total steroid (i.e., the sum of androstenedione and the unknown steroid) from DHA-4-^{14}C.

The average rate of formation of U.V. absorbing steroids by the two normal glands studied was 4.0 µg. per minute per mg. nitrogen and of the five hyperplastic glands 4.79 ± 0.51 µg. There seems to be a slight increase in hyperplastic glands, but

TABLE XXXII

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Normal and Hyperplastic Human Adrenal Glands

The data recorded in this table are derived from Tables XXX and XXXI

Gland number	Type of gland	mg. nitrogen per ml. of homogenate	Isolated Steroids: Androstenedione µg.	Isolated Steroids: Peak III µg.	Isolated Steroids: Total UV absorbing steroids µg.	µg. UV absorbing steroid formed per minute per mg. nitrogen
28	Normal	1.0	34	27	61	4.06
29	Normal	1.25	21	53	74	3.95
30	Hyperplasia	2.0	71	54	125	4.13
31	Hyperplasia	1.07	36	39	75	4.63
32	Hyperplasia	1.10	37	46	83	5.03
33	Hyperplasia	1.35	66	18	84	4.15
34	Adenomatous hyperplasia	1.20	59	38	97	5.39
		1.20	30	59	89	4.94
		1.20	30	65	95	5.28

no statistics can be applied due to the small number of normal glands for comparison. A similar problem in interpretation existed when unlabelled DHA was used as the substrate (Table XXVII), but some of the values found in those hyperplastic glands also fell within the normal range. However, the possibility exists that there is an increase in the activity of the Δ_5-3βHSD system for DHA in hyperplastic glands.

Two further steroid peaks were eluted on alumina column chromatography of the incubation extracts (peaks IV and V). In glands 28 and 31 (Table XXXIII), neither was demonstrable, but in the remainder they composed between 1.41% and 16.6% of the utilized substrate, peak IV always containing more radio-activity than peak V. No reliable ultra-violet absorption curves were obtained in the corresponding fraction upon spectrophotometry of these fractions. It was concluded that they did not possess the Δ_4-3-ketone chromophore grouping absorbing at 240 mμ and consequently were not steroids formed by the action of the Δ_5-3βHSD system upon the substrate DHA.

Attempts to isolate ^{14}C-labelled 11β-hydroxyandrostenedione, adrenosterone or testosterone by paper chromatography of the appropriate column fractions after the addition of authentic carrier steroids were unsuccessful in each assay. No radioactivity could be detected in carrier steroids after the paper chromatographic step.

TABLE XXXIII

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Normal and Hyperplastic Human Adrenal Glands

Incubation conditions as in Table XXX

Gland number	Type of gland	Isolated Steroids			
		Peak IV*		Peak V*	
		Tcpm⌿	% of utilized substrate	Tcpm⌿	% of utilized substrate
28	Normal	-	-	-	-
29	Normal	250	5.6	120	2.6
30	Hyperplasia	4,310	5.3	1,003	1.2
31	Hyperplasia	-	-	-	-
32	Hyperplasia	310	1.6	146	0.74
33	Hyperplasia	434	1.1	120	0.31
34	Adenomatous hyperplasia	850	5.3	195	1.2
		1,208	12.3	419	4.3
		910	6.3	205	1.4

* see Fig. 38b.

⌿ Total counts per minute

Table XXXIV compares the activity of the Δ_5-3βHSD system for DHA as measured by the silica gel and alumina column procedures. It can be seen that the difference of the two means, found for hyperplastic glands, is just statistically significant ($P < 0.5 > 0.4$), indicating a somewhat higher steroid recovery with alumina chromatography.

To establish further this apparent difference, three separate incubation extracts, two with DHA-4-^{14}C as substrate, one of which contained 732 µg. of androstenedione added to the incubate as a trap, and one with unlabelled DHA as substrate, all from the same gland, were chromatographed initially using the silica gel column technique and the combined fractions eluted from this column were rechromatographed on an alumina column.

The results obtained are tabulated in Table XXXV. The apparent losses incurred when the results of the silica gel and alumina columns are compared are probably due to the fact that the recovery of standards from an alumina column is only 90% In addition losses would occur due to combining the silica gel column fractions and to plating one fortieth of each sample for counting.

As the total cpm and the total U.V. absorbing steroids recovered after the two procedures closely match one another (Table XXXV) it can be concluded that there is no true difference

TABLE XXXIV

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Comparison of Silica Gel and Alumina Column Chromatographic Procedures

The data recorded in this table are derived from Tables XXVII and XXXII

Type of gland	Column chromatographic procedure	Number of glands	μg. UV absorbing steroid formed per minute per mg. nitrogen mean $\pm$ S.D.	Probability (*t* test)
Normal	Silica Gel	2	4.18	-
	Alumina	2	4.00	
Hyperplasia	Silica Gel	5	4.57 ± 0.40	$< 0.5 > 0.4$
	Alumina	5	4.79 ± 0.51	

TABLE XXXV

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY

Comparison of results obtained by Silica Gel and Alumina Column Chromatography

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 1.6 ml. of 20% (W/V) homogenate.
Additions: NAD, 6 mg. Final Volume 1.8 ml.
Substrate: Assay 1 - DHA-4-^{14}C, 130 µg., specific activity 479 cpm/µg.
Assay 2 - DHA-4-^{14}C, 130 µg., specific activity 479 cpm/µg. with added androstenedione, 732 µg.
Assay 3 - DHA, 130 µg.
Incubation Time: 30 minutes. Gas-phase: air.

Assay sample number	Silica Gel Column Chromatography		Alumina Column Chromatography				Percentage UV absorbing steroid recovered from alumina column	Percentage Tcpm recovered from alumina colum
	Total UV absorbing steroid µg.	Tcpm eluted	Androstenedione µg.	Peak III µg.	Total UV absorbing steroid µg.	Tcpm eluted		
1	64.9	55,074	14.2	41.0	54.2	44,826	83	81
2	727.7	49,398	550.5	23.3	573.8	35,550	79	74
3	66.2	-	39.5	12.3	51.8	-	78	-

between the two methods. However, the use of an alumina column is preferable due to its greater resolution in the separation of the formed steroids. The two steroids measured by the silica gel column procedure are mainly androstenedione and that of peak III of the alumina column.

UNKNOWN STEROID (PEAK III) ELUTED FROM ALUMINA COLUMNS

Almost all the experiments reported in this thesis were conducted before the identity of this steroid was established. Due to the small quantities of the steroid produced in each incubation, unequivocal identification of the substance by infra-red spectrophotometry could not be achieved until several hundred µg, were collected from a number of separate incubations.

As the steroid absorbed U.V. light and gave a positive dinitrobenzene reaction (Zimmermann chromagen), it was inferred that the substance was a 17-ketosteroid possessing a Δ_4-3-ketone grouping and thus probably arising in the androstenedione formed from the DHA of the assay procedure. In addition, the specific activity of this substance calculated from either the U.V. absorbancy or from the Zimmermann chromagen was identical.

Although peak III was eluted from an alumina column in a more polar effluent fraction than was authentic 11β-hydroxyandrostenedione, it was thought that this represented its most probable identity. When carrier 11β-hydroxyandrostenedione, testosterone, and adrenosterone were added to this radio-active unknown steroid and chromatographed in the A system of Bush, the radio-activity did not separate from authentic 11β-hydroxyandrostenedione. No radio-activity was detected in the well-separated paper spots of

either testosterone or adrenosterone (Table XXXVI).

However, in the B_3 and B_1 system of Bush, separation of the radio-activity from carrier 11β-hydroxyandrostenedione was readily achieved, all of the radio-activity being located as a discrete zone more polar than the 11β-hydroxyandrostenedione.

Further attempts to characterise this steroid were made in the Bush B_1 system but it separated from each of the following carrier steroids: 6β-hydroxyandrostenedione, 16α-hydroxy-androstenedione, 19-hydroxyandrostenedione (Table XXXVI), which were considered to be the hydroxylated androstenedione derivatives most likely to be formed.

The steroid was acetylatable with acetic anhydride and pyridine (Bush, 1961) and the acetate thus formed possessed a chromatographic mobility similar to the acetates of both DHA and testosterone in the Bush A system and ligroin-propylene glycol system of Savard. This indicated the presence of at least one easily acetylatable hydroxyl group. A positive result was obtained using the soda fluorescence technique for Δ_4-3-ketones on paper by a sample of the free steroid after paper chromatography.

All of this evidence agreed with the interpretation that the unknown compound was a 17-ketosteroid possessing a Δ_4-3-ketone group with at least one acetylatable hydroxyl group.

Its absorption spectrum was determined in ethanol-sulphuric

TABLE XXXVI

PAPER CHROMATOGRAPHY OF UNKNOWN STEROID

(Peak III of Alumina Column Chromatography)

		Paper Chromatographic Systems		
		Bush A Rf.	Bush B_3 Rf.	Bush B_1 Rf.
Unknown		0.05*	0.28	0.25
	11β-hydroxyandrostenedione	0.05*	0.33	0.48
	Adrenosterone	0.14	0.54	-
	Testosterone	0.15	0.55	-
	DHA	0.30	0.72	-
	Androstenedione	0.47	0.78	-
	6β-hydroxyandrostenedione	-	-	0.30
	16α-hydroxyandrostenedione	-	-	0.27
	19-hydroxyandrostenedione	-	-	0.20

*Separates from all authentic compounds on admixture except from 11β-hydroxyandrostenedione in Bush A system

acid (Smith & Bernstein, 1963). Authentic androstenedione, testosterone and 11β-hydroxyandrostenedione scanned concurrently gave absorption spectra which were identical with those reported by other workers (Smith & Bernstein, 1963). The absorption spectrum of the peak III substance was - λ maximum, 288 mμ and 360 mμ; λ minimum, 310 mμ and 400 mμ. These values did not correspond with any other hydroxylated androstenediones reviewed (Smith & Bernstein, 1963).

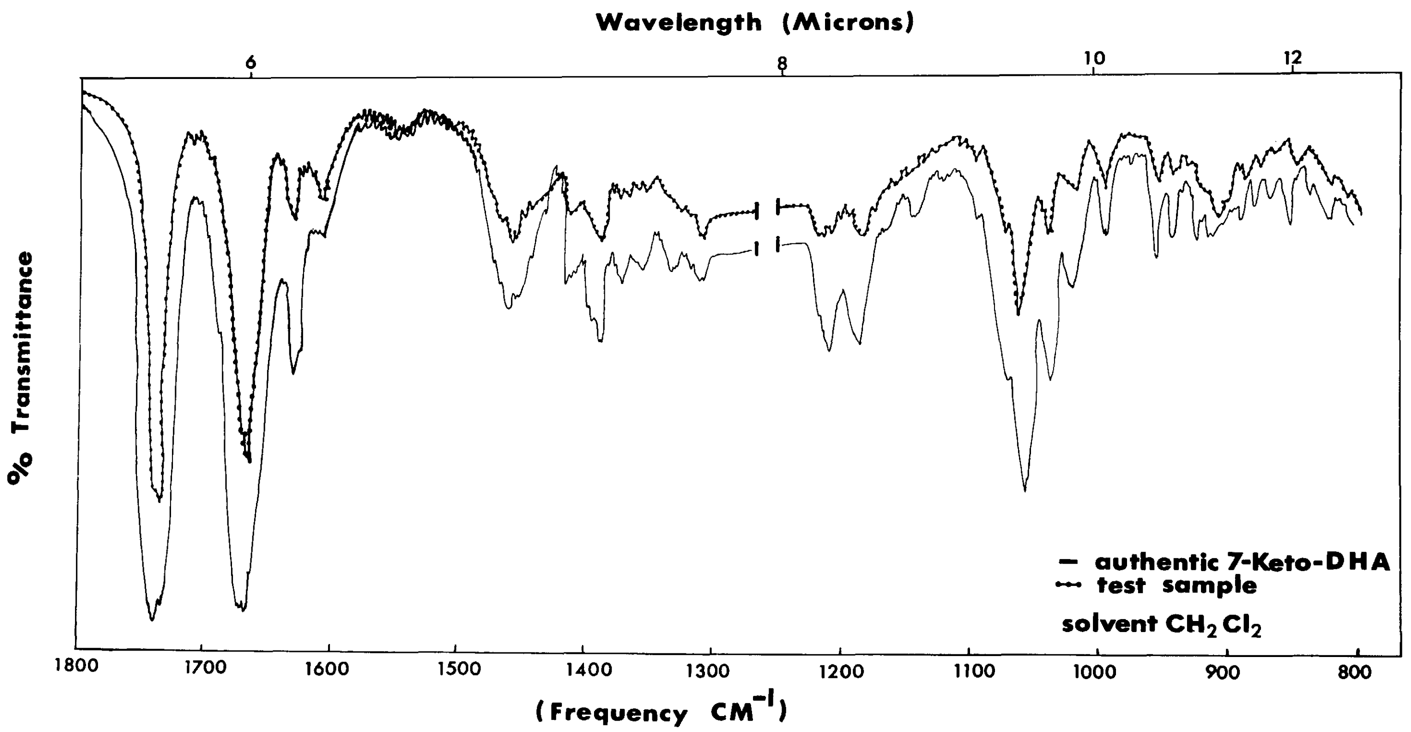
It was decided that only infra-red spectrophotometry would establish its identity. Further incubations were necessary to accumulate sufficient steroid for this characterisation and in the course of these incubations, the unknown compound was found to be formed not only from DHA-4-^{14}C, but also, in small amounts, from androstenedione-4-^{14}C and unlabelled androstenedione. 400 μg. of the unknown compound were amassed from these experiments and the combined steroid sample submitted, derived from both DHA and androstenedione, showed only one zone of radio-activity as judged by column and paper chromatography.

The infra-red spectroscopy was kindly conducted by Dr. K.D. Roberts and Dr. S. Lieberman of the Departments of Biochemistry and of Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University, New York.

The infra-red spectrum of the unknown steroid is shown in

Figure 39. It is identical to that of a sample of authentic 7-keto-dehydroepiandrosterone (7-keto-DHA) tested concurrently.

The presence of a Δ_5-7-ketone grouping explains the finding of its absorbing U.V. light, and giving a positive soda fluorescence reaction (Bush, 1961). As it is a 17-ketosteroid it would give a positive dinitrobenzene reaction (Zimmermann chromagen) and by having a β-hydroxyl group at position 3, it would yield a mono-acetate on acetylation with acetic acid and pyridine.



<u>Fig. 39</u>.- Infra-red spectrum.

UNKNOWN STEROIDS (PEAKS IV & V) ELUTED FROM ALUMINA COLUMNS

The compounds corresponding to peaks IV and V of the alumina columns may represent either intermediates in the formation of or further derivatives of 7-keto-DHA or androstenedione. Neither absorbs U.V. light, but the steroid (peak IV) gave a positive dinitrobenzene reaction (Zimmermann chromagen) while the steroid composing peak V did not.

STEROID CORRESPONDING TO PEAK IV

The compound was highly polar and possessed an Rf of 0.44 in the Bush B_5 system (cortisol, Rf. 0.25). This corresponds closely to the Rf value of 0.42 reported by Stárka and Katová (1962) for 7α-hydroxy-DHA in this system.

The unknown steroid was easily acetylatable using the conditions of DeCourcy and his colleagues (1953), following which, the steroid had a paper chromatographic mobility similar to that of DHA-acetate. The 7α-hydroxy group is also acetylated under these conditions (Burnstein et al., 1959). Saponification of the acetate(s) using methanolic potassium bicarbonate failed to release the free steroid, but this was achieved with methanolic sodium hydroxide. Since similar observations have been noted when saponification of DHA-acetate is attempted, this may indicate the presence of a 3β-hydroxy group. Being capable of forming a

Zimmermann chromagen the substance is probably a 17-ketosteroid and when considered in relation to the polarity of other steroids in the Bush B_5 system, it is possible that this substance is 7α-hydroxy-DHA. Definitive proof must await the availability of an authentic standard (in preparation) and the amassing of sufficient material from further incubations for an infra-red spectrum to be obtained.

STEROID CORRESPONDING TO PEAK V

This compound failed to move from the starting line in the Bush B_5 system, but was easily acetylatable, following which it possessed an Rf of 0.33 in this paper system (Cortisol, Rf. 0.25). Saponification failed using methanolic potassium bicarbonate, but with methanolic sodium hydroxide, two products were obtained, one of which had a paper chromatographic mobility similar to the steroid corresponding to peak IV (0.44). The second product possessed an Rf. of 0.60 in the Bush B_5 system. The identity of this compound (peak V) is completely unknown, but it may be a 17-hydroxylated steroid with a Δ_5-3β-hydroxy group due to the negative Zimmermann reaction, no U.V. light absorbtion at 240 mμ and the failure to saponify with methanolic potassium bicarbonate.

REINTERPRETATION OF THE RESULTS OF THE ASSAY OF THE Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE USING DHA-4-^{14}C

In the light of finding that the unknown steroid (peak III) is 7-keto-DHA and not a Δ_4-3-ketosteroid, a reappraisal of the results of the various assays of the Δ_5-3βHSD system for DHA is essential. Using this experimental model, two principal products are formed by human adrenal glands in <u>in vitro</u> incubations using DHA-4-^{14}C as substrate, namely androstenedione and 7-keto-DHA. The formation of androstenedione represents a true assessment of the activity of the Δ_5-3βHSD system. The formation of 7-keto-DHA by adrenal tissue has not been shown previously under <u>in vitro</u> conditions. The relationship of the 7-keto-DHA formation to the Δ_5-3βHSD system is not clear.

The modified results are tabulated and summarised in Table XXXVII. The activity of both enzyme systems remains very variable not only between glands but also in any one gland (Gland 34, Table XXXVII). Gland 29 (Table XXXVII) shows a very low level of androstenedione formation and a high level of 7-keto-DHA formation. However, despite the variation in the figures from the other normal gland (Gland 28) the recovery of total cpm added, isolated as DHA,androstenedione, and 7-keto-DHA was 85%. Of interest is the finding that the sum of the amounts of androstenedione and 7-keto-DHA formed in each incubation of the same homogenate of

TABLE XXXVII

UTILIZATION OF DHA-4-^{14}C BY NORMAL AND HYPERPLASTIC HUMAN ADRENAL GLANDS

The data recorded in this table are derived from Table XXXII

Gland number	Type of gland	Isolated Steroids			
		Androstenedione µg.	µg. androstenedione formed per minute per mg. nitrogen	7-keto-DHA µg.	µg. 7-keto-DHA formed per minute per mg. nitrogen
28	Normal	34	2.26	27	1.80
29	Normal	21	1.12	53	2.83
30	Hyperplasia	71	2.36	54	1.80
31	Hyperplasia	36	2.24	39	2.40
32	Hyperplasia	37	2.24	46	2.79
33	Hyperplasia	66	3.25	18	0.90
34	Adenomatous hyperplasia	59	3.26	38	2.13
		30	1.67	59	3.26
		30	1.67	65	3.61

Summary

Type of gland	Number of glands	Δ_5-3β-hydroxysteroid Dehydrogenase System	7-keto-DHA System
		µg. androstenedione formed per minute per mg. nitrogen mean ± S.D.	µg. 7-keto-DHA formed per minute per mg. nitrogen mean ± S.D.
Normal	2	1.69	2.31
Hyperplasia	5	2.38 ± 0.66	2.41 ± 0.92

gland 34 (Tables XXXII & XXXVII) fall within the same range despite the variation in the proportion of the two steroids formed.

The Δ_5-3βHSD activity in hyperplastic glands is 2.38 ± 0.66 µg. androstenedione per minute per mg. nitrogen, whereas in normal tissues, it is 1.69 µg. (Table XXXVII). Statistical evaluation of these results cannot be performed due to the small normal sample but due to the overlapping of the values, it is doubtful whether any difference in the activity of this enzyme system exists between normal and hyperplastic tissues. Similarly, the activity of the enzyme system(s) leading to the formation of 7-keto-DHA from DHA is similar in both types of tissue.

This is the first demonstration of the formation of 7-keto-DHA *in vitro* by the human adrenal cortex. Biochemical assay of the human adrenal Δ_5-3βHSD system has been performed by others using similar incubation conditions to that in the present investigation and with DHA as substrate (Rubin *et al*., 1961; Bloch *et al*., 1962; Rubin *et al*., 1963). Assays of normal and neoplastic adrenals have been made and the results have been expressed as µg. androstenedione formed per minute per mg. nitrogen. These results were obtained by direct spectrophotometric comparison of incubates against tissue blanks. It seems possible that not only androstenedione but also 7-keto-DHA have been measured and included in the figures for

Δ_5-3βHSD activity. These reported results are all higher than those presented here, but they also show considerable variation between one gland and another (Rubin et al., 1961; Bloch et al., 1962; Rubin et al., 1963).

7-keto-DHA was isolated in small amounts from the urine of both normal patients and those with a variety of adrenal hyperfunctional disorders (Lieberman et al., 1948) and characterised subsequently (Fukushima et al., 1954). Gallagher (1958) suggested that it may represent a product of adrenal biosynthesis rather than a peripheral metabolite of DHA, and he showed that it occurred in elevated amounts in two patients with adrenal carcinomas causing Cushing's syndrome. Baulieu (1962) found 7-keto-DHA sulphate in adrenal tumours associated with both virilism and Cushing's syndrome and noted its occurrence in elevated concentrations in the peripheral plasma and in the urine. Because adrenal venous plasma contained the sulphate ester of 7-keto-DHA in higher concentrations than peripheral plasma, he concluded that it was indeed a product of the adrenal gland. No free 7-keto-DHA was demonstrated in any of the tumours, in plasma or in urine, so that conjugation appeared to occur in the adrenal itself.

The 7-keto-DHA, isolated in the present experiments is the free unconjugated steroid. Between 85% and 95% of the total cpm. added as substrate DHA were recovered after column chromatography.

Never more than 1% of the total cpm. added as substrate DHA were found in the aqueous residue of the incubation remaining after extraction. Thus no significant sulphation occurred under the assay conditions.

MECHANISM OF 7-KETO-DHA FORMATION

Despite the evidence presented on page 169, for the active formation of 7-keto-DHA by the human adrenal cortex, the possibility that it represents an auto-oxidation product of DHA must be excluded. 7-keto-cholesterol and the 7α and 7β-hydroxycholesterols have been shown to be products of the non-enzymatic oxidation of cholesterol (Wintersteiner & Bergström, 1941; Bergström, 1943). Cholesterol may also be oxidized to 7-keto-cholesterol when exposed in thin films to air and irradiated with U.V. light (Windaus _et al._, 1941) or when present in aqueous solutions and exposed to X-rays (Keller & Weiss, 1950; Weiss & Keller, 1950). To ensure that 7-keto-DHA occurred as a true biosynthetic product of DHA, the following experiment was performed.

METHODS

A 10% (W/V) homogenate of a human autopsy adrenal gland was made with equal volumes of 0.154M sodium chloride and 0.1M phosphate buffer. 30 mg. of NAD and 3724 mg. of DHA-4-^{14}C (specific activity 64.8 cpm/μg) were added to each of three flasks. 10 ml. of medium alone were pippetted into one flask. 10 ml. of the 10% (W/V) homogenate added to a second flask and to the final flask, 10 ml. of the 10% (W/V) homogenate which had been boiled.

The incubates were shaken in air at 37°C for 60 minutes.

2 volumes of acetone were added to each flask and the resultant clear supernatent, following filtration and removal of the acetone *in vacuo*, was extracted three times with 5 volumes of benzene: chloroform (6:1). The pooled extracts were taken to dryness and submitted to alumina column chromatography. The appropriate column fractions corresponding to the polarity of 7-keto-DHA were combined and rechromatographed on paper in the Bush B_1 system.

RESULTS AND DISCUSSION

No evidence of the formation of 7-keto-DHA in any of the incubates could be detected by alumina column chromatography (Table XXXVIII) or by paper chromatography. No peak of radioactivity or U.V. light absorption could be found in the areas corresponding to the polarity of 7-keto-DHA. Thus the formation of 7-keto-DHA can be assumed to be the result of a biosynthetic process of the adrenal cortex.

Micro-organisms have been shown to be capable of converting DHA to 7α and 7β-hydroxy-DHA and 7-keto-DHA (Dodson *et al.*, 1959); cholesterol to 7-hydroxycholesterol (Kramli & Horvath, 1948, 1949); and pregnenolone to 7α and 7β-hydroxypregnenolone (Epstein *et al.*, 1956). Δ_4-3-ketosteroids can also serve as substrate for bacterial hydroxylation at C-7 in that 7α-hydroxy-11-deoxycortisol and 7α-hydroxy-androstenedione were formed by the micro-organism

TABLE XXXVIII

MECHANISM OF 7-KETO-DHA FORMATION

Alumina Column Chromatography

Isolated steroids	Incubate		
	Chloride-phosphate buffer only + DHA-4-^{14}C Tcpm	Boiled human autopsy adrenal tissue + DHA-4-^{14}C Tcpm	Human autopsy adrenal tissue + DHA-4-^{14}C Tcpm
Androstenedione	-	-	-
DHA	203,950	189,439	180,737
7-keto-DHA	-	-	-
Peak IV	-	-	-
Peak V	-	-	-
Recovery (as DHA)	84.5%	78.5%	74.9%

Cephalosporium sp. (Bernstein et al., 1959).

Recently, rat liver homogenates have been found to be capable of forming 7α-hydroxy-DHA (Starka & Katova, 1962) and the 7α-hydroxylation of oestrone has been reported to be effected by beef adrenals (Knuppen et al., 1964). In both these reports, the possibility of the 7-oxygenated steroids being auto-oxidation products was excluded.

These studies together with those involved in the isolation of 7-keto-DHA from human urine, plasma and adrenal venous blood (p. 169) point to it being an adrenal product, and that the formation of 7-keto-DHA represents another biosynthetic pathway in the adrenal cortex, the presence of which now appears to be established by the in vitro investigations reported here.

UTILIZATION OF DHA-4-^{14}C BY NORMAL AND HYPERPLASTIC HUMAN ADRENAL GLANDS

EFFECTS OF VARIATIONS IN THE AMOUNTS OF TISSUE INCUBATED AND IN THE INCUBATION MEDIUM

An alteration in the incubation medium, from the sodium chloride-phosphate medium to KRPG with added nicotinamide, was made to see if any change occurred in the proportions or rate of androstenedione and 7-keto-DHA formation from DHA. In other experiments using either type of medium, the amounts of tissue incubated and the period of the incubation were both increased to raise the chances of detecting steroids formed in trace amounts.

METHODS

0.5 ml. of a 10% (W/V) homogenate of an hyperplastic gland, in KRPG with added 0.01M nicotinamide, was incubated (Table XXXIX). Using between 1.5 ml. and 2.5 ml. of 10% (W/V) homogenate, comparison of the two types of media were made. The periods of incubation varied from 30 to 90 minutes. The detailed incubation, conditions are included in the appropriate tables (Table XL & XLI). Extraction and alumina column chromatography were performed as described.

TABLE XXXIX

UTILIZATION OF DHA-4-^{14}C BY AN HYPERPLASTIC HUMAN ADRENAL GLAND

Gland Number - 35   Gland Weight 10.0 g.

Medium: KRPG with added 0.01M nicotinamide
Tissue: 0.5 ml. of 10% (W/V) homogenate
Additions: NAD, 6.0 mg.   Final Volume - 0.7 ml.
Substrate: DHA-4-^{14}C, 200 μg., specific activity 180 cpm/μg.
Incubation Time: 30 minutes.   Gas-phase: air.

Isolated steroids	Assay Number			
	1		2	
	μg.	Specific activity cpm./μg.	μg.	Specific activity cpm./μg.
DHA	40	176	45	179
Androstenedione	51	170	18	180
7-keto-DHA	36	175	57	180

Isolated steroids	Assay Number			
	1		2	
	Tcpm	% of utilized substrate	Tcpm	% of utilized substrate
Peak IV	1,202	8.0	710	4.7
Peak V	1,080	8.0	820	6.2

Isolated steroids	Average μg. formed	μg. formed per minute per mg. nitrogen
Androstenedione	35	1.91
7-keto-DHA	47	2.58

TABLE XL

UTILIZATION OF DHA-4-^{14}C BY NORMAL AND HYPERPLASTIC HUMAN ADRENAL GLANDS

Varying Tissue Concentrations and Incubation Times

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Gas-phase: - air.

Gland number	Type of gland	Gland weight g.	Tissue: 10% (W/V) homogenate		NAD mg. added	Substrate: DHA-4-^{14}C		Incubation time min.
			ml.	mg.		µg. added	Specific activity (cpm/µg)	
36	Normal	3.50	2.0	200	24	500	90	90
37	Hyperplasia	8.30	2.5	250	30	400	220	60
38	Hyperplasia	10.0	1.5	150	12	200	100	90

Isolated steroids	Gland Number					
	36		37		38	
	µg.	Specific activity cpm/µg.	µg.	Specific activity cpm/µg.	µg.	Specific activity cpm/µg.
DHA	201	90	151	210	84	97
Androstenedione	123	83	82	226	74	98
7-keto-DHA	151	88	95	224	42	94

Isolated steroids	Gland Number					
	36		37		38	
	Tcpm	% of utilized substrate	Tcpm	% of utilized substrate	Tcpm	% of utilized substrate
Peak IV	1,910	8.1	5,089	12.8	961	9.3
Peak V	1,260	5.4	2,210	5.5	615	5.8

Gland number	Androstenedione		7-keto-DHA	
	µg. formed	µg. formed per minute per mg. nitrogen	µg. formed	µg. formed per minute per mg. nitrogen
36	123	0.50	151	0.62
37	82	0.55	95	0.64
38	74	0.46	42	0.26

TABLE XLI

UTILIZATION OF DHA-4-^{14}C BY NORMAL AND HYPERPLASTIC HUMAN ADRENAL GLANDS

Varying Amounts of Tissue and Incubation Times

Medium: KRPG with added 0.01M nicotinamide
Gas-phase: Air.

Gland number	Type of gland	Gland weight g.	Tissue 10% (W/V) Homogenate		NAD mg. added	Substrate DHA-4-^{14}C		Incubation time min.
			ml.	mg.		µg. added	Specific activity cpm/µg.	
39	Normal	3.50	2.0	200	24	500	90	90
40	Hyperplasia	10.0	1.5	150	12	200	100	90

Isolated steroids	Gland Number			
	39		40	
	µg.	Specific activity cpm/µg.	µg.	Specific activity cpm/µg.
DHA	91	85	77*	98
Androstenedione	108	90	47*	95
7-keto-DHA	154	87	62*	99

Isolated steroids	Gland Number			
	39		40	
	Tcpm	% of utilized substrate	Tcpm	% of utilized substrate
Peak IV	2,725	11.7	1,990*	8.6
Peak V	892	8.5	527*	5.1

Gland number	Androstenedione		7-keto-DHA	
	µg. formed	µg. formed per minute per mg. nitrogen	µg. formed	µg. formed per minute per mg. nitrogen
39	108	0.44	154	0.63
40	47*	0.30	62*	0.38

* Average values of three incubations

RESULTS AND DISCUSSION

No difference in the rate of formation of androstenedione or 7-keto-DHA from DHA-4-^{14}C was seen when the hyperplastic gland was incubated in KRPG (Table XXXIX) as compared to the sodium chloride-phosphate medium (Table XXXVII). Both steroids were derived only from the added DHA, as shown by comparison of their specific activities with that of the substrate. Considerable variation in the proportion of the two steroids formed may be seen (Table XXXIX).

The substance comprising peaks IV and V of the alumina column were found again in this experiment making up a somewhat higher percentage of the utilized substrate when the incubation was performed in KRPG (Tables XXXIII & XXXIX).

Increasing the period of the incubation and the amount of tissue did not lead to any other steroid being found in detectable amounts under those conditions (Table XL). The activity of both the Δ_5-3βHSD system and the enzyme system(s) responsible for the formation of 7-keto-DHA, calculated as µg. formed per minute per mg. nitrogen, is lower for the longer periods (60-90 minutes) of incubation than found for lower tissue concentrations incubated for 30 minutes (Table XL). This is possibly due to a falling off of the rate of DHA utilization with time. However, activity/time experiment was not done.

The percentage of the recovered total cpm found in the steroid fractions corresponding to peaks IV and V of the alumina column is similar to that noted with lower tissue concentrations and/or shorter incubation times (Tables XXXIII & XXXIX).

Essentially similar findings were noted when large tissue concentrations were incubated for longer periods of time using KRPG as the medium. Only androstenedione and 7-keto-DHA were formed in significant quantities and their specific activities corresponded closely to those of the recovered substrate DHA indicating that they were derived only from the added substrate (Table XLI).

The activity of the Δ_5-3βHSD system for DHA and the 7-keto-DHA system show a similar decrease in overall activity per unit time as was noted with the increased tissue concentrations incubated in the sodium chloride-phosphate medium. This fall in activity occurs despite a proportional increase in the NAD present with the high tissue amounts, when compared with the activity in the 50 mg. tissue incubations.

The normal gland (Gland 36, Table XL; Gland 39, Table XLI) and the hyperplastic gland (Gland 38, Table XL; Gland 40, Table XLI) are portions of the same gland homogenized and incubated in the different media cited. While no difference can be detected in the rate of formation of the two major products by the normal gland, the activity of the Δ_5-3βHSD system appears to be less in the

KRPG incubate of the hyperplastic gland (Table XLII). There is a corresponding increase in the rate of formation of 7-keto-DHA, the sum of the two products formed being the same for both media.

The eluates from the alumina columns, of assays using either KRPG or sodium chloride and phosphate buffer, corresponding to the zones in which authentic 11β-hydroxyandrostenedione, testosterone, and adrenosterone would be expected (Shoulder of Peak II), were re-chromatographed in the paper systems of Bush after the addition of appropriate authentic steroids as carrier. Constant specific activity could not be achieved for any of these carrier steroids; all radio-activity was lost on repeated chromatography of the carrier steroids.

TABLE XLII

UTILIZATION OF DHA-4-^{14}C BY NORMAL AND HYPERPLASTIC HUMAN ADRENAL GLANDS

Comparison of Media

The data recorded in this table are derived from Tables XL and XLI

Type of gland	Incubation time min.	Medium: Sodium Chloride and Phosphate Buffer		Medium: KRPG	
		μg. androstenedione formed per minute per mg. nitrogen	μg. 7-keto-DHA formed per minute per mg. nitrogen	μg. androstenedione formed per minute per mg. nitrogen	μg. 7-keto-DHA formed per minute per mg. nitrogen
Normal	90	0.50	0.62	0.44	0.63
Hyperplasia	90	0.46	0.26	0.30	0.38

THE FORMATION OF 11β-HYDROXYANDROSTENEDIONE AND TESTOSTERONE FROM DHA-4-^{14}C BY NORMAL AND HYPERPLASTIC HUMAN ADRENAL GLANDS

In order to confirm that only the four compounds described were formed on the utilization of DHA-4-^{14}C by normal and hyperplastic glands, the extracts of several incubates were submitted to paper chromatography without a prior column chromatographic step. It was thought that any other trace steroids might be detected more readily using this technique.

METHODS

The extracts were initially chromatographed for 8 hours in the Bush B_1 system. The over-flows ("run-offs") from each strip contained DHA, androstenedione, 11β-hydroxyandrostenedione, testosterone and adrenosterone as judged by comparison with the mobility of authentic standard samples. The more polar steroids, including 7-keto-DHA, remained on the Bush B_1 paper chromatograms.

To these overflows, authentic carrier 11β-hydroxyandrostenedione testosterone and adrenosterone were added and the whole sample containing the carrier steroids was then rechromatographed for 15 hours in the Bush A system. Excellent separation of the carrier steroids was achieved. DHA and androstenedione, recovered from the overflows of this second chromatogram, were chromatographed for a third time in the Bush A system for a period of 6 hours. No

significant amounts of radio-activity were found in the overflows of the third, Bush A, paper chromatograms.

Following radiochromatogram scanning of each of the paper chromatograms, the zones of radio-activity corresponding to DHA, androstenedione and 7-keto-DHA were eluted, counted and measured by either the Zimmermann reaction or spectrophotometry.

The U.V. absorbing zones corresponding to 11β-hydroxy-androstenedione, testosterone and adrenosterone were eluted from the papers, measured at 240 mμ in the spectrophotometer and their specific activities calculated after counting.

Testosterone acetate was made at room temperature with equal volumes of acetic anhydride and pyridine for 15 hours (DeCourcy _et al_., 1953). The product was chromatographed on the Bush A system, and the specific activity of the derivative determined after elution. Subsequent saponification with alcoholic potassium bicarbonate (Meyer, 1953) was performed and the free testosterone was rechromatographed in the Bush B_3 system and the specific activity again determined.

11β-hydroxyandrostenedione and adrenosterone were rechromatographed for a second and third time using the Bush B_1 and B_3 systems. Their specific activities were determined after each procedure. No derivatives of these two steroids were made. Constancy of specific activity in three paper systems was taken as evidence of their radiochemical purity.

RESULTS

The radiochromatogram scans revealed the presence of five peaks of radio-activity of which three corresponded to androstenedione, DHA and 7-keto-DHA. Two highly polar discrete zones of radio-activity were noted near the origin of the strips run in the Bush B_1 system and corresponded to the unknown steroids of peaks IV and V of the alumina columns. Neither appeared to absorb U.V. light. The specific activities of the isolated DHA, androstenedione and 7-keto-DHA agreed within 10% in every instance (Table XLIII). An average of 75% of the added substrate was recovered as unchanged DHA, androstenedione and 7-keto-DHA from the various chromatograms.

No radio-activity was detected by radiochromatogram scanning in the chromatographic areas corresponding to the carrier steroids, 11β-hydroxyandrostenedione, adrenosterone and testosterone. However, four of the six glands examined formed trace amounts of 11β-hydroxyandrostenedione, three of these glands (Glands 43, 44 & 45, Table XLIV) being hyperplastic and removed from patients with Cushing's syndrome. In one case (Gland 45, Table XLIV), traces of testosterone were also detected. None of the glands formed adrenosterone.

TABLE XLIII

UTILIZATION OF DHA-4-^{14}C BY NORMAL AND HYPERPLASTIC HUMAN ADRENAL GLANDS

Incubation Extracts Subjected Directly to Paper Chromatography

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 0.5 ml. of 10% (W/V) homogenate
Additions: NAD, 6 mg. Final Volume: 0.7 ml.
Substrate: DHA-4-^{14}C, 130 µg. specific activity either *345 cpm/µg. or **479 cpm/µg. (***Gland 42 - 200 µg., specific activity, 175 cpm/µg.)
Incubation Time: 30 minutes. Gas-phase: air.

Gland number	Type of gland	Isolated Steroids					
		DHA		Androstenedione		7-keto-DHA	
		µg.	Specific activity cpm/µg.	µg.	Specific activity cpm/µg.	µg.	Specific activity cpm/µg.
41*	Normal	25	346	30	309	13	339
42***	Hyperplasia	98	181	31	163	31	167
43*	Hyperplasia	21	320	29	316	31	330
44*	Hyperplasia	12	348	13	331	60	361
45**	Hyperplasia	15	475	49	470	14	470
46**	Hyperplasia	67	478	22	452	9	458

TABLE XLIV

UTILIZATION OF DHA-4-^{14}C BY NORMAL AND HYPERPLASTIC HUMAN ADRENAL GLANDS

Formation of 11β-hydroxyandrostenedione, Testosterone and Adrenosterone

Incubation conditions are as shown in Table XLIII

Gland number	Type of gland	Isolated Carrier Steroids with Constant Specific Activity		
		11β-hydroxyandrostenedione cpm/µg.	testosterone cpm/µg.	adrenosterone cpm/µg.
41	Normal	1.4	nil	nil
42	Hyperplasia	nil	nil	nil
43	Hyperplasia	3.8	nil	nil
44	Hyperplasia	4.5	nil	nil
45	Hyperplasia	4.6	0.25	nil
46	Hyperplasia	nil	nil	nil

THE ROUTES OF FORMATION OF 7-KETO-DHA

Following the investigation of the Δ_5-3βHSD system for DHA in normal and hyperplastic glands using small amounts of tissue, several distinct problems remained.

The identity of the radio-active steroid corresponding to peak III of the alumina column elutions was unknown at this time. It was assumed to be a 17-ketosteroid possessing a Δ_4-3-ketone grouping, most probably derived from androstenedione. It had been shown not to be 11β-hydroxyandrostenedione, 6β-hydroxyandrostenedione, 16α-hydroxyandrostenedione, or 19-hydroxyandrostenedione, but to possess at least one hydroxyl group capable of acetylation. Its source from either androstenedione or DHA had not been established. It was thought that, if incubations using DHA-4-^{14}C as the substrate in the presence of unlabelled androstenedione as a trap were done, the specific activity of the isolated 7-keto-DHA would give some indication of the relative importance of the two substrates as sources of the compound.

The trace amounts of 11β-hydroxyandrostenedione and testosterone detected in several incubations required further clarification and definitive establishment of their production.

It was towards those two problems that three incubations were planned, one with a normal gland, and the other two with hyperplastic glands. DHA-4-^{14}C alone or in the presence of an androstenedione

trap and androstenedione-4-^{14}C were chosen as substrates.

METHODS

Gland 47 - Normal Adrenal Gland.

3.0 ml. of a 10% (W/V) homogenate, with KRPG containing 0.01M nicotinamide as the medium, were incubated with either 260 µg. DHA-4-^{14}C or 1000 µg. of androstenedione-4-^{14}C as substrates for 90 minutes in air. No NAD was added. The incubates were extracted with 5 volumes benzene:chloroform (6:1), taken to dryness and subjected directly to paper chromatography using the B_1 system of Bush with a running time of 8 hours. The zone of radio-activity, found by radiochromatogram scanning of the paper, corresponding to the "unknown" (7-keto-DHA) was eluted and chromatographed on a 7.5 g. alumina column. The overflows of those paper chromatograms, containing androstenedione, DHA and substances of the polarity of 11β-hydroxyandrostenedione, were chromatographed on a separate 7.5 g. alumina column.

Gland 48 - Hyperplastic Adrenal Gland.

Three separate flasks containing 3.0 ml. of a 10% (W/V) homogenate in KRPG with added 0.01M nicotinamide were incubated with 30 mg. of NAD added for 90 minutes in air. DHA-4-^{14}C was added as substrate to two flasks, one of which also contained 732 µg. of unlabelled androstenedione as a trap. The remaining

flask contained 1,000 µg. of androstenedione-4-^{14}C as the substrate. The flasks were extracted in a similar manner to gland 47 and chromatographed on a 7.5 g. alumina column.

Gland 49 - Hyperplastic Adrenal Gland.

KRBG with added 0.01M nicotinamide was the incubation medium. 5.0 ml. of a 10% (W/V) homogenate were incubated with either 260 µg. of DHA-4-^{14}C or 1,000 µg. of androstenedione-4-^{14}C for 120 minutes in the presence of 30 mg. of NAD. The gas-phase was 95% oxygen-5% carbon dioxide. The extraction and chromatographic procedures were identical with those of gland 48.

RESULTS AND DISCUSSION

The results obtained by the incubation of the normal gland with DHA and androstenedione are shown in Table XLV. Separate columns for the isolation of 7-keto-DHA and the less polar 17-ketosteroids were run. The significant result is the finding of 7-keto-DHA as a product of androstenedione-4-^{14}C. Only 3.3 µg. of 7-keto-DHA were formed from this substrate, while 33µg. were derived from DHA. Consequently, the formation of 7-keto-DHA occurs more readily from DHA.

The incubation of the hyperplastic gland (Gland 48) shows that both androstenedione and 7-keto-DHA were formed from DHA-4-^{14}C . Using androstenedione-4-^{14}C as substrate, 22µg. of 7-keto-DHA were

TABLE XLV

UTILIZATION OF DHA-4-^{14}C AND ANDROSTENEDIONE-4-^{14}C BY A NORMAL HUMAN ADRENAL GLAND (GLAND 47)

Medium: KRPG with added 0.01M nicotinamide
Tissue: 3.0 ml. of 10% (W/V) homogenate
Additions: Nil. Final volume: 3.0 ml.
Substrate: 1) DHA-4-^{14}C, 260 µg. specific activity 479 cpm/µg.
2) Androstenedione-4-^{14}C, 1,000 µg. specific activity 455 cpm/µg.
Incubation Time: 90 minutes. Gas-phase: air.

Column peaks	Isolated steroids	Substrate: DHA-4-^{14}C			Substrate: Androstenedione-4-^{14}C		
		Tcpm	µg.	Specific activity cpm/µg.	Tcpm	µg.	Specific activity cpm/µg.
I	Androstenedione	43,318	94	460	359,632	806	446
II	DHA	47,756	103	463	2,266	-	-
Shoulder of II	*	2,949	-	-	1,602	-	-
III	7-keto-DHA	15,573	33	474	1,470	3.3	440

* Substances of the polarity of 11β-hydroxyandrostenedione, testosterone and adrenosterone

formed, compared with 39 µg. from the DHA substrate flask (Table XLVI). In the presence of the androstenedione trap, 125 µg. 7-keto-DHA were produced (Table XLVI). From the ratio of the specific activities of the isolated 7-keto-DHA and DHA (44.5 cpm/µg: 65.0 cpm/µg., Table XLVI), it is possible to calculate that 68.6% of the total 7-keto-DHA, formed in this flask, came directly from DHA-4-^{14}C, the remainder coming from the androstenedione, present as a trap, which diluted the 7-keto-DHA formed from DHA itself and caused the observed drop in specific activity.

The increase in the quantity of 7-keto-DHA, in the presence of androstenedione, would appear to be due to amarked decrease in the conversion of DHA to androstenedione. The 125 µg. of 7-keto-DHA is twice the amount formed from DHA and androstenedione when they were incubated separately. This agrees with a decrease of 59% in the total cpm. incorporated into androstenedione when the incubates with DHA-4-^{14}C and DHA-4-^{14}C and androstenedione trap are compared (Table XLVI).

39 µg. of 7-keto-DHA were formed from DHA-4-^{14}C when incubated alone (Table XLVI). Approximately twice the total cpm. were incorporated into 7-keto-DHA when DHA-4-^{14}C and unlabelled androstenedione were incubated together. If the assumption is made that 78 µg. (i.e., twice the 39 µg. formed from DHA alone) came from DHA-4-^{14}C in the trapping incubation, based on the

TABLE XLVI

UTILIZATION OF DHA-4-^{14}C, IN THE PRESENCE OF ANDROSTENEDIONE AND ANDROSTENEDIONE-4-^{14}C BY A HYPERPLASTIC HUMAN ADRENAL GLAND (GLAND 48)

Medium: KRPG with added 0.01M nicotinamide
Tissue: 3.0 ml. of a 10% (W/V) homogenate
Additions: NAD, 30 mg. Final Volume: 4.0 ml.
Substrate: 1) DHA-4-^{14}C, 538 μg., specific activity, 64.8 cpm/μg.
2) DHA-4-^{14}C, 538 μg., specific activity, 64.8 cpm/μg. with added 732 μg. unlabelled androstenedione
3) Androstenedione-4-^{14}C, 1,000 μg., specific activity, 455 cpm/μg.
Incubation Time: 90 minutes. Gas-phase: air.

Column peaks	Isolated steroids	Substrate: DHA-4-^{14}C			Substrate: DHA-4-^{14}C with added 732 μg. androstenedione			Substrate: Androstenedione-4-^{14}C		
		Total cpm	μg.	Specific activity cpm./μg.	Total cpm	μg.	Specific activity cpm./μg.	Total cpm	μg.	Specific activity cpm./μg.
I	Androstenedione	16,632	264	63	6,951	720	9.3	452,317	902	501
II	DHA	10,087	155	65	14,227	219	65	3,230	-	-
Shoulder of II	*	800	-	-	542	-		2,211	-	-
III	7-keto-DHA	2,448	39	63	5,578	125	44.6	11,441	22	513
IV	-	3,280	-	-	3,425	-		2,650	-	-
V	-	752	-	-	1,099	-		2,465	-	-

* Substances of the polarity of 11β-hydroxyandrostenedione, testosterone and adrenosterone

TABLE XLVII

UTILIZATION OF DHA-4-^{14}C and ANDROSTENEDIONE-4-^{14}C BY A HYPERPLASTIC HUMAN ADRENAL GLAND (GLAND 49)

Medium: KRBG with added 0.01M nicotinamide
Tissue: 5.0 ml. of a 10% (W/V) homogenate
Additions: NAD, 30 mg. Final Volume: 6.0 ml.
Substrate: 1) DHA-4-^{14}C, 260 µg., specific activity 479 cpm/µg.
2) Androstenedione-4-^{14}C, 1,000 µg., specific activity 455 cpm/µg.
Incubation Time: 120 minutes. Gas-phase: 95% O_2 and 5% CO_2

Column Fractions	Isolated steroids	Substrate: DHA-4-^{14}C			Substrate: Androstenedione-4-^{14}C		
		Tcpm	µg.	Specific activity cpm/µg.	Tcpm	µg.	Specific activity cpm/µg.
I	Androstenedione	106,538	250	426	327,131	747	483
II	DHA	4,274	9	450	1,474	-	-
Shoulder of II	*	936	-	-	3,610	-	-
III	7-keto-DHA	1,752	4	441	501	-	-
IV	-	306	-	-	-	-	-
V	-	192	-	-	-	-	-

* Substances of the polarity of 11β-hydroxyandrostenedione, testosterone and adrenosterone

At this stage in the investigations, the finding that the "unknown" substance (peak III, alumina columns: subsequently established as 7-keto-DHA) could be formed from androstenedione further supported the assumption that it was a 17-ketosteroid with a Δ_4-3-ketone grouping.

The accumulated "unknown" fractions from these incubations with DHA-4-^{14}C, DHA-4-^{14}C with unlabelled androstenedione as a trap and androstenedione-4-^{14}C were pooled and added to the corresponding fractions isolated from previous DHA-4-^{14}C incubations. This total sample was chromatographed twice in the paper systems, Bush B_1 and B_3, followed by a further alumina column fractionation, from which a single peak-fraction was obtained. It was this fraction containing the steroid derived from both sources, i.e., DHA and androstenedione, which was examined by infra-red spectrophotometry. The tracing was identical with authentic 7-keto-DHA (Fig. 39). Thus it seems assured that androstenedione can be converted to 7-keto-DHA.

Previous experiments, using various tissue and substrate concentrations and incubation periods, have shown that the rate of formation of the sum of androstenedione and 7-keto-DHA from DHA did not differ in normal and hyperplastic human adrenal glands. The difference in the rate of formation of androstenedione and 7-keto-DHA by the normal and hyperplastic glands (Glands 47 & 48)

might be due to the omission of NAD from the incubation of Gland 47 (Table XLVIII). Thus, not only the Δ_5-3βHSD system but also the enzyme system(s) for the formation of 7-keto-DHA from DHA may require the presence of NAD. The fall in the <u>rate</u> of formation of androstenedione from DHA by the hyperplastic gland 49 (Table XLVIII) may be related to the different medium the longer period of incubation and the larger amounts of tissue compared with Gland 48 (Tables XLVI-XLVIII).

The formation of the two more polar compounds, corresponding to peaks IV and V of the alumina column eluants, were formed by the hyperplastic gland (Gland 48, Table XLVI) not only from DHA-4-^{14}C but also from androstenedione-4-^{14}C. Peak IV could be 7α-hydroxy-DHA as judged by its chromatographic mobilities. Thus its formation from DHA-4-^{14}C in the presence of an androstenedione trap in similar amounts to that from DHA-4-^{14}C alone, is to be expected, if one assumes it to be an intermediate in the 7-keto-DHA pathway.

These two compounds were formed in only trace amounts as was 7-keto-DHA in the presence of KRBG as the medium (Gland 49, Table XLVII).

TABLE XLVIII

UTILIZATION OF DHA-4-^{14}C AND ANDROSTENEDIONE-4-^{14}C BY NORMAL AND HYPERPLASTIC HUMAN ADRENAL GLANDS

The data recorded in this table are derived from Tables XLV, XLVI and XLVII

Gland number and type	Substrate	Isolated Steroids			
		Androstenedione		7-keto-DHA	
		µg. formed	µg. formed per minute per mg. nitrogen	µg. formed	µg. formed per minute per mg. nitrogen
47 normal	DHA-4-^{14}C	94	0.333	33	0.085
	Androstenedione-4-^{14}C	-	-	3.3	0.0085
48 hyperplasia	DHA-4-^{14}C	264	0.913	39	0.135
	DHA-4-^{14}C with androstenedione	107	0.366	86*	0.30*
				39≠	0.136≠
	Androstenedione-4-^{14}C	-	-	22	0.076
49 hyperplasia	DHA-4-^{14}C	250	0.263	4	0.004
	Androstenedione-4-^{14}C	-	-	1	0.001

* µg. 7-keto-DHA formed from DHA-4-^{14}C

≠ µg. 7-keto-DHA fromed from androstenedione. These values were calculated from the specific activities and counts incorporated into 7-keto-DHA (see page 193)

THE ROUTES OF FORMATION OF 11β-HYDROXYANDROSTENEDIONE, ADRENOSTERONE AND TESTOSTERONE

HUMAN ADRENAL GLANDS

The appropriate alumina column fractions containing 11β-hydroxyandrostenedione, testosterone and adrenosterone, as judged by the concurrent column chromatography of authentic samples of these steroids, were re-chromatographed in the paper systems of Bush after the addition of the respective carrier steroids and the substances with added carrier isolated. The procedures involved have been outlined previously (p. 185). Constancy of specific activity after three steps on paper was taken as the index of radiochemical purity.

The normal adrenal gland (Gland 47, Table XLIX) was found to form 11β-hydroxyandrostenedione, adrenosterone and testosterone from both DHA-4-^{14}C and androstenedione-4-^{14}C. They were formed in only trace amounts. Taken together as a group, they accounted for 0.5% of the total cpm of the incubated substrate DHA and 0.04% of the incubated androstenedione cpm.

The higher incorporation of cpm from DHA than androstenedione suggested that DHA was a more important precursor than androstenedione. However, the hyperplastic gland associated with Cushing's syndrome (Gland 48, Table XLIX) incorporated more radio-activity into 11β-hydroxyandrostenedione and adrenosterone when DHA-4-^{14}C alone

TABLE XLIX

UTILIZATION OF DHA-4-^{14}C AND ANDROSTENEDIONE-4-^{14}C BY NORMAL AND HYPERPLASTIC HUMAN ADRENAL GLANDS

The Formation of 11β-hydroxyandrostenedione, Adrenosterone and Testosterone

Incubation conditions are as shown in Tables XLV, XLVI and XLVII

100 μg. of each authentic steroid added as carrier to the appropriate column fractions.

Gland number	Type of gland	Substrate	Isolated Steroids: 11β-hydroxy-androstenedione Tcpm	11β-hydroxy-androstenedione %* conversion	Adrenosterone Tcpm	Adrenosterone % conversion	Testosterone Tcpm	Testosterone % conversion
47	Normal	DHA-4-^{14}C	327	0.26	160	0.13	148	0.12
		Androstenedione-4-^{14}C	94	0.02	31	0.007	69	0.015
48	Hyperplasia	DHA-4-^{14}C	160	0.45	47	0.13	97	0.28
		DHA-4-^{14}C with added unlabelled androstenedione	99	0.28	27	0.08	116	0.33
		Androstenedione-4-^{14}C	567	0.13	88	0.02	22	0.005
49	Hyperplasia	DHA-4-^{14}C	Not detected		Not detected		Not detected	
		Androstenedione-4-^{14}C	653	0.14	368	0.08	2515	5.7

* % of total cpm. added as substrate

was the substrate than when an unlabelled androstenedione trap also was added. If androstenedione was the major precursor, then the total cpm. isolated in those two substances should be much less in the presence of the trap than was actually found. The total cpm incorporated from DHA into testosterone was elevated in the presence of unlabelled androstenedione and only 22 cpm. were derived from androstenedione-4-^{14}C.

In Gland 49 (Table XLIX) no radio-activity could be detected in any of these carrier steroids when DHA-4-^{14}C served as the substrate, but approximately 6% of the substrate androstenedione-4-^{14}C was converted principally to testosterone.

These experiments confirm that trace amounts of these three steroids may be formed from DHA-4-^{14}C under the present conditions. In the normal gland and one of the hyperplastic glands (Gland 48) it is possible that DHA is a more important source than androstenedione as judged by comparison of the total cpm. incorporated. This is particularly true for testosterone and is evidence for their formation in some glands, directly from DHA and not from androstenedione.

BOVINE ADRENAL GLANDS (BOVINE GLAND 1)

Bovine adrenal glands are known to be capable of producing *in vitro* 11β-hydroxyandrostenedione, testosterone and adrenosterone.

An experiment was designed to test the possibility that these three steroids are formed from DHA directly in this tissue.

METHODS

25 ml. of a 10% (W/V) homogenate of bovine adrenal glands, prepared in equal volumes of 0.154M sodium chloride and 0.1M phosphate buffer, were incubated in air for 90 minutes. 60 mg. of NAD were added to a final volume of 27 ml. in each flask. Three separate flasks were established. In flask 1, 1,700 µg. of DHA-4-^{14}C (specific activity 125 cpm/µg.) was the substrate. 1,700 µg. of the DHA-4-^{14}C and 1,464 µg of unlabelled androstenedione were the steroid additions to flask 2 while 1,984 µg. of androstenedione-4-^{14}C (specific activity, 145 cpm/µg.) and 1,750 µg of unlabelled DHA served as the substrates for flask 3. The incubates were extracted with benzene:chloroform 6:1 (3 times 5 volumes), and the extracts pooled and taken to dryness.

Alumina column chromatography was performed as described previously (p. 146). The androstenedione formed, after counting and measurement, was chromatographed twice in the Bush A system, constant specific activity of the eluted steroid being achieved.

The appropriate fractions of the alumina columns, containing 11β-hydroxyandrostenedione, testosterone and adrenosterone, were chromatographed on the Bush A, B_3 and B_1 systems. Their separation

was achieved using the Bush A system and constant specific activity of each steroid was obtained following elution, counting and measurement from each of the three paper systems. Their identities were established by comparison with the mobilities of authentic steroids run concurrently. No derivatives were made.

RESULTS

The results are tabulated in Table L. The specific activities of 11β-hydroxyandrostenedione, testosterone and adrenosterone are the same as that for androstenedione in flask 1 in which DHA-4-^{14}C alone was the substrate. By contrast in flask 2 in which unlabelled androstenedione was present their specific activities are lower than that of androstenedione indicating utilization of the unlabelled steroids while when androstenedione-4-^{14}C was the substrate in the presence of unlabelled DHA, their specific activities are higher than the isolated androstenedione, which was diluted by unlabelled androstenedione formed from DHA, the higher specific activities of these three steroids indicating utilization of the androstenedione-^{14}C.

Consequently, 11β-hydroxyandrostenedione, testosterone and adrenosterone are formed by the *in vitro* incubation of bovine adrenal glands by a pathway which involves androstenedione. No evidence for a direct pathway from DHA is obtained, pointing to a

TABLE L

UTILIZATION OF DHA-4-^{14}C AND ANDROSTENEDIONE-4-^{14}C BY A BOVINE ADRENAL GLAND (BOVINE GLAND 1)

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 25 ml. of a 10% (W/V) homogenate
Additions: NAD, 60 mg. Final Volume: 27 ml.
Substrate: Flask 1 - DHA-4-^{14}C, 1,700 µg., specific activity, 125 cpm/µg.
Flask 2 - DHA-4-^{14}C, 1,700 µg., specific activity 125 cpm/µg. and unlabelled androstenedione, 1,464 µg
Flask 3 - Androstenedione-4-^{14}C, 1,984 µg., specific activity, 145 cpm/µg. and unlabelled DHA, 1,750 µg.
Incubation Time: 90 minutes. Gas-phase: air.

Isolated steroids	Flask 1		Flask 2		Flask 3	
	Tcpm	Specific activity cpm/µg.	Tcpm	Specific activity cpm/µg.	Tcpm	Specific activity cpm/µg.
Androstenedione	181,838	114	169,890	75	233,202	78
11β-hydroxyandrostenedione	7,536	110	3,084	31	22,272	118
Adrenosterone	3,072	112	1,032	31	6,096	114
Testosterone	2,490	110	1,000	32	4,354	124

difference between their formation by bovine and human adrenal tissue *in vitro* from DHA.

FORMATION OF DHA-4-^{14}C FROM ANDROSTENEDIONE-4-^{14}C BY A NORMAL HUMAN ADRENAL GLAND (GLAND 47)

Following alumina column chromatography of the incubate of Gland 47 with androstenedione-4-^{14}C, a peak of radio-activity was noted associated with the mobility of authentic DHA (Table XLV). If the substance was found to be DHA, this would be evidence for reversibility of the Δ_5-3βHSD system for DHA to androstenedione in human adrenal glands.

The appropriate column fractions were submitted to paper chromatography in the Bush A system (running time, 6 hours) with authentic androstenedione and DHA run concurrently as standards. Most of the radio-activity possessed the same Rf as androstenedione, but a small proportion corresponded to the mobility of DHA. This zone was eluted and 200 μg. of DHA added as carrier. The sample was then rechromatographed in the Ligroin-Propylene glycol system of Savard and after elution the DHA was measured by the Zimmermann reaction and counted, allowing its specific activity to be estimated.

The sample was next acetylated and the DHA-acetate run on the Bush A system. After elution, counting and measurement, it was saponified to the free alcohol and run once more in the Bush A system, eluted, measured and counted. Constancy of specific activity was taken as evidence of radiochemical purity.

A known amount of DHA-4-^{14}C was taken through identical procedures after the addition of DHA to the test sample. From this, a recovery of 30% of the total cpm. added was found after the paper chromatographic steps and acetylation procedures which corresponded well with the final 61 μg. of DHA recovered from the 200 μg. originally added as carrier (31% recovery).

The results are tabulated in Table LI, from which it can be seen that DHA of constant specific activity was isolated. The specific activity of 2.1 cpm/μg. found after the second paper chromatogram altered little on acetylation and subsequent saponification of the acetate. The total cpm. isolated as DHA (corrected for overall recovieries) represented a conversion of 0.1% of the substrate androstenedione-4-^{14}C or 8.6% of the utilized substrate.

During the course of these investigations, Ward and Engel (1964) published a report in which sheep adrenal microsomes were shown to convert androstenedione-4-^{14}C to DHA in the presence of NADH. This conversion did not proceed in the presence of NAD. The omission of NAD from the incubations of the normal human adrenal gland explains why DHA formation from androstenedione was found only in this experiment. In Glands 48 and 49, extracts of both of the incubations with androstenedione-4-^{14}C as substrate did not contain DHA after the various procedures outlined above were

TABLE LI

FORMATION OF DHA-4-^{14}C FROM ANDROSTENEDIONE-4-^{14}C BY GLAND XLVII

Incubation Conditions are Shown in Table XLV

200 µg. authentic DHA added as carrier after the first paper chromatogram of the appropriate column fraction

Procedure	Derivative	Paper chromatographic systems	DHA-4-^{14}C		
			µg. present*	Tcpm	Specific activity cpm./µg.
-	-	Bush A	200	801	4.0
-	-	Ligroin - propylene glycol	150	315	2.1
Acetylation	DHA-acetate	Bush A	80	176	2.2
Saponification to free steroid	DHA	Bush A	61	140	2.3

* µg. DHA determined by Zimmermann reaction

performed. In both, NAD had been added to the incubation in a concentration of 30 mg. per incubate. Only negligable counts were detected in the DHA areas after two paper chromatograms of the extracts of these incubates. These counts disappeared on acetylation of the eluted zones.

However, since 7-keto-DHA can be formed from androstenedione in the presence of NAD (Tables XLV, XLVI & XLVII), the reversal of the reaction, androstenedione to DHA, would appear to be a different mechanism from that involving 7-keto-DHA formation in which the presence of NADH is not obligatory.

IN VITRO STUDY OF A BENIGN VIRILISING ADRENAL TUMOUR (GLAND 27)

Virilising tumours of the adrenal cortex are rare causes of either the adrenogenital syndrome or Cushing's syndrome. While bilateral adrenocortical hyperplasia causing the adrenogenital syndrome has been shown, principally by urinary studies, to be due to a deficiency of one of the adrenal enzyme systems responsible for the stepwise hydroxylation of the steroid molecule, most commonly the 21-hydroxylase (Jailer _et al_., 1955; Bongiovanni, 1958) and less frequently either the 11β-hydroxylase (Eberlein & Bongionvanni, 1956) or the Δ_5-3βHSD (Bongiovanni, 1961), the relationship of these enzyme systems to the causation of virilism by adrenocortical tumours is little understood. Two tumours causing virilism have been shown to be deficient in Δ_5-3βHSD activity for DHA (Gland 26 & 27, Table XXVIII) as measured by _in vitro_ studies presented here.

Lipsett & Wilson (1962) studied 10 functioning adrenal tumours associated with both the "mixed" type of Cushing's syndrome and virilism and found, from urinary studies, evidence of defective 11β-hydroxylation in nine, and impaired 21-hydroxylase and Δ_5-3βHSD activity in another two, both of which were associated only with virilism. Previously, a deficiency of the Δ_5-3βHSD had been postulated as a cause of the high DHA urinary excretion in some adrenal tumours (Rubin & Dorfman, 1957).

Goldman and his colleagues (1964) demonstrated, histochemically and by in vitro incubation studies, the absence of the Δ_5-3βHSD in 5 of 6 adrenal tumours causing virilism. A relative deficiency of this enzyme system has been reported in this condition by Rubin and her associates (1963). 17α-hydroxypregnenolone was converted in vitro to DHA more easily by an adrenal tumour than by normal adrenal glands (Solomon et al., 1960). Thus an abnormality of the Δ_5-3βHSD appears to exist in some of the virilising adrenal tumours. With the demonstration that several substrate-specific Δ_5-3βHSD systems exist in bovine adrenal tissue (Ewald et al., 1964a, 1964b, 1964c; Kowal et al., 1964a, 1964b; Krüskemper et al., 1964), a deficiency of the Δ_5-3βHSD system specific for DHA could not only explain the increased amounts of urinary DHA in adrenal virilism, but also help to explain the occurrence of virilism alone in the presence of the secretion of normal amounts of C_{21} Δ_4-3-ketosteroids. Whether the DHA produced by the tumour is responsible wholly for the virilism is not known as nearly all is present as the sulphate conjugate in adrenal and peripheral venous plasma (Baulieu, 1962), but DHA and DHA-sulphate are freely interconvertible (Roberts et al., 1961).

In 1962, the opportunity arose to study in vitro the biosynthetic pathways of a 228 g. adrenal tumour removed from a boy, aged 8 years, with precocious homologous pseudopuberty. The

significant plasma and urinary steroid values are shown in Table LII. The clinical and urinary biochemical findings have been the subject of a recent report (Mortimer _et al._, 1964).

Due to the high urinary DHA levels found, the possibility that the Δ_5-3βHSD system for DHA was defective in this tumour was surmised and incubations were planned to assay the Δ_5-3βHSD for DHA and also to determine the activity of the Δ_5-3βHSD systems acting upon either pregnenolone or 17α-hydroxypregnenolone.

METHODS

The Δ_5-3βHSD system was assayed in duplicate using the modification of the method of Rubin and her colleagues (1961), previously described (p. 128). Other aliquots of the homogenate were incubated with DHA-4-^{14}C as the substrate, these extracts being submitted directly to paper chromatography using the Bush A, B_1 and B_5 systems.

A 30% (W/V) homogenate of a portion of the tumour was also prepared in KRPG containing 0.01M nicotinamide, and 3.0 ml. of this homogenate was incubated with cholesterol-4-^{14}C in the presence of 30 mg. of NAD for 90 minutes in air. To one of the three incubates, 200 µg. of pregnenolone were added, while to another, 250 µg. of progesterone were added, both to serve as trapping agents.

TABLE LII

PLASMA AND URINARY STEROID VALUES OF PATIENT

WITH VIRILISING ADRENAL TUMOUR (GLAND 27)⨍

	Normal values (mg./day)	Values Found in Patient (mg/day)	
		Preoperatively	3 months postoperatively
Urinary 17-ketosteroids	10	290	2.5
Urinary DHA	0.2-4.5	254	not measured
Urinary 17-ketogenic steroids	10	20-25	12
Plasma cortisol	5-15*	10-15*	16*

⨍ Mortimer, Rudd and Butt (1964)
* μg./100 ml.

The incubates which contained cholesterol-4-^{14}C as substrate were extracted after dilution with 2 volumes of water with 3 volumes of benzene:chloroform (6:1), and the extracts taken to dryness and then partitioned between 50% aqueous methanol and hexane. Paper chromatography of the residue of the methanol partition fraction was performed in the systems of Bush, Zaffaroni and Savard. Five steroids were tentatively identified after repeated chromatography. To each, suitable carrier steroid was added, followed by further paper and silica gel column chromatography. Derivatives were formed and constancy of specific activity after several steps was taken as evidence of radiochemical purity.

RESULTS

The assay procedure using DHA as substrate failed to reveal any ultraviolet absorbing steroids following silica gel column chromatography (Table LIIIa, see also Gland 27, Table XXVIII). Loss of ultra-violet absorbing steroids was excluded by the chromatography, directly on paper, of duplicate extracts with DHA-4-^{14}C as substrate. No ultra-violet absorbing areas were detected and no radio-activity was found in the zones corresponding to the mobilities of androstenedione or 7-keto-DHA (Table LIIIb), thus indicating the absence of the Δ_5-3βHSD system for DHA and the system(s) for 7-keto-DHA formation under these conditions.

TABLE LIII

Δ_5-3β-HYDROXYSTEROID DEHYDROGENASE ASSAY IN

A VIRILISING ADRENAL TUMOUR (GLAND 27)

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 1.6 ml. of 20% (W/V) homogenate
Additions: NAD, 6 mg. Final Volume: 1.8 ml.
Substrate: DHA, 500 µg.
Incubation Time: 30 minutes. Gas-phase: air.

A Silica Gel Chromatography

Gland weight g.	Number of samples assayed	µg. UV absorbing steroid formed	mg. nitrogen per ml. homogenate	µg. UV absorbing steroid formed per minute per mg. nitrogen
228	2	Nil	2.79	Nil

* This result has been recorded previously (Gland 27, Table XXXVIII

Incubation conditions as shown in section A except
that substrate was DHA-4-^{14}C, 180 µg., specific activity, 608 cpm/µg.

B Duplicate Samples Put Directly on Paper

Number of samples assayed	Isolated Steroids			mg. nitrogen per ml. homogenate	µg. UV absorbing steroid formed per minute per mg. nitrogen
	DHA specific activity cpm./µg.	Androstenedione µg.	7-keto-DHA µg.		
2	479	Nil	Nil	2.79	Nil

The specific activity of the isolated radio-active DHA from both incubation flasks was found to have decreased from the 608 cpm/µg. of the added substrate to 478 cpm/µg. and 480 cpm/µg. respectively. From the ratio of the specific activities of the substrate isolated DHA-4-^{14}C (608 cpm/µg.:479 cpm/µg., Table LIIIB), it is possible to calculate the dilution of the substrate during the period of incubation. A minimum of 48 µg. of unlabelled DHA was contributed to the DHA pool during the incubation period from endogenous sources by 320 mg. of tumour homogenate.

Consequently, a minimum of 5.0 µg. of DHA were produced per minute per g. net weight of tumour _in vitro_, so that if the entire tumour had been functioning constantly at this rate _in vivo_, 1.64 g. of DHA could have been produced per day. However, the urinary output was of the order of 254 mg. per day (Table LII). The difference between the two values may be due to the _in vitro_ conditions, and that large portions of the tumour were inactive due to necrosis and haemorrhage.

When portions of the tumour homogenate were incubated with cholesterol-4-^{14}C, five steroids possessing a Δ_4-3-ketone grouping were isolated and identified following the addition of carrier steroids (Table LIV). 17α-hydroxyprogesterone was isolated from the flask to which no trapping agent was added, while deoxycorticosterone and corticosterone were identified from the incubation in which pregnenolone served as a trap. Deoxycorticosterone and cortisol

TABLE LIV

STEROIDS ISOLATED FROM INCUBATIONS OF A VIRILISING ADRENAL TUMOUR WITH CHOLESTEROL-4-^{14}C (GLAND 27)

Medium: KRPG with added 0.01M nicotinamide
Tissue: 3.0 ml. of 30% (W/V) homogenate
Additions: NAD, 30 mg. Final Volume: 4.0 ml.
Substrate:
1) cholesterol-4-^{14}C, 400 μg., specific activity, 635 cpm/μg.
2) cholesterol-4-^{14}C, 400 μg., specific activity, 635 cpm/μg. and unlabelled pregnenolone, 200 μg.
3) cholesterol-4-^{14}C, 400 μg., specific activity, 635 cpm/μg. and unlabelled progesterone, 250 μg.

Incubation Time: 90 minutes. Gas-phase: air.

Isolated steroid	Procedures	Steroid derivative	Chromatography		Specific activity cpm./mg.
			Paper	Column	
17α-hydroxy-progesterone	-	-	Bush A	-	7,570
	-	-	Bush B_3	-	1,716
	-	-	-	Silica Gel	1,595
	Oxidation	Androstenedione	Bush A	-	1,600
Deoxycortico-sterone* (DOC)	-	-	Bush A	-	356
	Acetylation	DOC acetate	Bush A	-	310
	Saponification	DOC	Ligroin - propylene glycol	-	356
Deoxycortico-sterone** (DOC)	-	-	Bush A	-	410
	Acetylation	DOC acetate	Bush A	-	397
	Saponification	DOC	Ligroin - propylene glycol	-	392
Corticosterone (compd. B)*	-	-	Bush B_1	-	630
	Acetylation	B acetate	Bush A	-	688
	-	B acetate	Ligroin - propylene glycol	-	665
	Saponification	Corticosterone	Bush B_1	-	659
Cortisol** (compd. F)	-	-	Bush B_5	-	646
	Acetylation	F acetate	Bush B_1	-	757
	Saponification	Adrenosterone	Ligroin-propylene glycol	-	708

* Pregnenolone trap present
** Progesterone trap present

were isolated from the remaining flask containing the progesterone trap. Neither pregnenolone nor progesterone served as efficient traps, little of either added steroid being recovered.

The identification of these steroids possessing a Δ_4-3-ketone structure indicates the presence of an intact biosynthetic pathway from cholesterol to corticosteroids in the tumour. This is in agreement with the preoperative finding of elevated urinary 17-ketogenic steroids (Table LII). Thus a Δ_5-3βHSD system acting upon either pregnenolone or 17α-hydroxypregnenolone was present in the tumour in vivo and in vitro. However, as no equivalent system acting upon DHA could be demonstrated (Table LIII) the possible explanation lies in their being two Δ_5-3βHSD systems, one for either pregnenolone or 17α-hydroxypregnenolone and the other for DHA. This finding agrees with the suggestion by Weliky and Engel (1962) of multiple substrate-specific Δ_5-3βHSD systems in the human adrenal and with the finding that in beef and human adrenal tissue, the Δ_5-3βHSD can act upon a variety of Δ_5-androstenes and Δ_5-pregnenes in vitro (Berliner et al., 1962; Baulieu et al., 1936b; Kowal et al., 1964a; Pasqualini et al., 1964).

Two substrate specific Δ_5-3-ketosteroid isomerases have been found in beef adrenal glands (Ewald et al., 1964a, 1964b, 1964c; Krüskemper et al., 1964) and it was found possible to separate the Δ_5-pregnene-3,20-dione and the Δ_5-androstene-3,17-dione isomerases

(Ewald et al., 1964c). Two separate 3β-ol dehydrogenases for C_{19} steroids are thought to exist, as epiandrosterone had no competitive effect upon the conversion of DHA to androstenedione by beef adrenal acetone powders (Kowal et al., 1964b). Whether two separate 3β-ol dehydrogenases exist capable of acting upon the unsaturated 3β-hydroxysteroids remains to be elucidated.

The absence of the Δ_5-3βHSD system has been noted both biochemically in vitro and histochemically in other virilising adrenal tumours (Roversi et al., 1963; Goldman et al., 1964; p. 144).

Whether the entire Δ_5-3βHSD system for DHA was absent in the tumour just discussed, or whether only the isomerase or the dehydrogenase system was deficient cannot be stated since no intermediates of the overall reaction (i.e., Δ_5-androstene-3,17-dione or Δ_4-androstene-3β-ol-17-one) were incubated.

UTILIZATION OF DHA-4-^{14}C BY HUMAN AND BOVINE ADRENAL GLANDS IN THE PRESENCE OF PREGNENOLONE AND 17α-HYDROXYPREGNENOLONE

Since the evidence cited points to the existence of separate enzyme systems acting upon the Δ_5-pregnenes and Δ_5-androstenes, no substrate competition for the enzyme systems acting on DHA should be found when DHA and a Δ_5-3β-hydroxypregnene are incubated together. To determine if this was correct, incubations of homogenates prepared from surgically removed adrenal glands were done.

METHODS

Gland 50 - Hyperplastic Human Adrenal Gland.

Three separate flasks were prepared. 5.0 ml. of a 10% (W/V) homogenate, prepared in KRBG containing 0.01M nicotinamide and 30 mg. of NAD, were added to each flask. To two of the preparations either 6.0 mg. of pregnenolone or 5.0 mg. of 17α-hydroxypregnenolone were added. 272 µg. of DHA-4-^{14}C (specific activity, 485 cpm/µg.) were used as the substrate in all the flasks. The incubation time was 120 minutes, and the preparations were gassed with 95% oxygen and 5% carbon dioxide.

Gland 51 - Normal Human Adrenal Gland.

Three separate assays were prepared. 1.5 ml. of a 10% (W/V) homogenate, prepared in equal volumes of 0.154M sodium chloride and 0.1M phosphate buffer and containing 12 mg. of NAD, were added

to each flask. 850 µg. of DHA-4-^{14}C (specific activity 125 cpm/µg.) served as substrate in all three flasks, to two of which either 880 µg. of pregnenolone or 880 µg. of 17α-hydroxypregnenolone were added. The incubation time was 90 minutes and the gas-phase was air.

Normal Bovine Adrenal Gland (Bovine Gland 2).

Five separate flasks were established. 25 ml. of a 10% (W/V) homogenate, prepared in KRBG containing 0.01M nicotinamide and 60 mg. of NAD were added to each flask. 1,700 µg. of DHA-4-^{14}C (specific activity, 125 cpm/µg.) served as substrate in each incubation. To two of the flasks, either 2,200 µg. or 35.2 mg. of pregnenolone were added and to a further two, either 2,200 µg. or 35.2 mg. of 17α-hydroxypregnenolone were added. The incubation time was 90 minutes and the preparations were gassed with 95% oxygen and 5% carbon dioxide.

All the preparations were extracted three times with five volumes benzene:chloroform (6:1) after dilution with 2 volumes of water. The extracts were pooled and taken to dryness and chromatographed as previously described on a 7.5 g. alumina column for both human glands and a 10.0 g. alumina column for the bovine gland incubation.

RESULTS AND DISCUSSION

Using a KRBG medium, androstenedione had been found to be the principal product resulting from the utilization of DHA in a previous incubation (Gland 49, Table XLVII) and this was confirmed in the incubation of the hyperplastic gland (Gland 50, Table LV). The formation of 7-keto-DHA amounted to 1.5% of the recovered radio-activity in the control flask, 0.4% in the flask with added pregnenolone and 1.4% in that with added 17α-hydroxypregnenolone. The isolation of 7-keto-DHA was only possible from the tissue incubated with DHA alone.

The recoveries of total cpm., added as substrate, after column chromatography were 86% in the control flask, and 72% and 70% for the incubates with added pregnenolone and 17α-hydroxy-pregnenolone.

From Table LV, it can be seen that whereas 106,538 cpm. were incorporated into androstenedione from DHA, only 64,436 cpm. and 76,358 cpm. were formed in this fraction of the other incubates. Accordingly pregnenolone decreased the conversion of DHA to androstenedione by a factor of 39.5% and 17α-hydroxypregnenolone by a factor of 28.3%. These percentages would be reduced if allowance for the recovery of added substrate DHA-4-^{14}C is made, bringing the percentage decrease with pregnenolone down to 25% and 17α-hydroxy-pregnenolone to 12%. From the µg. of androstenedione recovered

TABLE LV

UTILIZATION OF DHA-4-^{14}C BY A HYPERPLASTIC HUMAN GLAND IN THE PRESENCE OF PREGNENOLONE AND 17α-HYDROXYPREGNENOLONE (GLAND 50)

Medium: KRBG with added 0.01M nicotinamide
Tissue: 5.0 ml. of 10% (W/V) homogenate
Additions: NAD, 30 mg. Final Volume: 6.0 ml.
Substrate: Assay 1:- DHA-4-^{14}C, 272 µg., specific activity, 485 cpm/µg.
Assay 2:- DHA-4-^{14}C, 272 µg., specific activity, 485 cpm/µg. with added pregnenolone, 6.0 mg.
Assay 3:- DHA-4-^{14}C, 272 µg., specific activity, 485 cpm/µg. with added 17α-hydroxypregnenolone, 5.0 mg.
Incubation Time: 120 minutes. Gas-phase: 95% oxygen, 5% carbon dioxide.

Column peak	Isolated steroids	Assay 1			Assay 2 (Pregnenolone)			Assay 3 (17α-hydroxypregnenolone)		
		Tcpm	µg.	Specific activity cpm/µg.	Tcpm	µg.	Specific activity cpm/µg.	Tcpm	µg.	Specific activity cpm/µg.
I	Androstenedione	106,538	250	426	64,436	161	400	76,358	246	310
II	DHA	4,274	9.5	450	29,520	63	469	13,042	39.5	330
Shoulder of II	Possible 11β-hydroxy-androstenedione testosterone, or adrenosterone	936	-	-	608	-	-	1,128	-	-
III	7-keto-DHA	1,752	4.0	438	402	-	-	1,314	-	-
IV	-	306	-	-	186	-	-	216	-	-
V	-	192	-	-	102	-	-	894	-	-

from each incubate, the uncorrected percentage inhibition amounted to 35.6% in the presence of pregnenolone, but only 1.5% in the case of 17α-hydroxypregnenolone. The reason for this latter discrepancy can be seen on comparison of the specific activities of the recovered androstenedione and DHA (Table LV).

The specific activity of DHA isolated from the pregnenolone flask is similar to that of the control, while the DHA from the 17α-hydroxypregnenolone flask is much lower. This is due to dilution of the added DHA-4-^{14}C substrate by unlabelled DHA derived by side chain cleavage of the 17α-hydroxypregnenolone by the tissue during the period of incubation. A total of 99 µg. of 17α-hydroxypregnenolone was converted to DHA. This is calculated from the ratio of the specific activities of the isolated DHA in the control and experimental flasks (450 cpm/µg.:330 cpm/µg.). The specific activities of the isolated androstenedione from the control and 17α-hydroxypregnenolone flasks are the same as those of the appropriate substrate DHA isolated. Pregnenolone reduced the amount of androstenedione formed from DHA and there is a fall in the specific activity of androstenedione as compared to the DHA substrate possibly due to some formation of androstenedione from pregnenolone _via_ 17α-hydroxypregesterone.

Thus in this hyperplastic gland (Gland 50) pregnenolone led to a significant inhibition in the conversion of DHA to androstenedione

while 17α-hydroxypregnenolone was without significant effect, the fewer cpm. found in the androstenedione fraction being due to dilution of the substrate DHA-4-^{14}C by unlabelled DHA derived from 17α-hydroxypregnenolone.

Table LVI presents the results of the utilization of DHA-4-^{14}C by a normal human adrenal gland in the presence of equimolar amounts of pregnenolone and 17α-hydroxypregnenolone. Both androstenedione and 7-keto-DHA were formed in each incubation. The 7-keto-DHA formed amounted to 2.9% of the recovered radio-activity in the control flask, 4.2% in the flask with added pregnenolone and 3.2% in that with added 17α-hydroxypregnenolone.

The recoveries of total cpm., added as substrate, after column chromatography were 93%, 94% and 93% in the control, pregnenolone and 17α-hydroxypregnenolone incubations.

It can be seen that similar amounts of radio-activity were incorporated into androstenedione from DHA, in the control (21,790 cpm) and 17α-hydroxypregnenolone (23,700 cpm.) flasks, while in the pregnenolone incubation, only 14,496 cpm. were found as androstenedione. This represents a decrease in the conversion of DHA to androstenedione of 33.5%. From the µg. of androstenedione recovered from each incubation, the percentage inhibition due to pregnenolone is 30%, while an apparent enhancement occured with 17α-hydroxypregnenolone, 12.5% more androstenedione being formed.

TABLE LVI

UTILIZATION OF DHA-4-^{14}C BY A NORMAL HUMAN GLAND IN THE PRESENCE OF

PREGNENOLONE AND 17α-HYDROXYPREGNENOLONE (GLAND 51)

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 1.5 ml. of 10% (W/V) homogenate
Additions: NAD, 12 mg. Final Volume: 1.9 ml.
Substrate: Assay 1 - DHA-4-^{14}C, 850 μg., specific activity, 125 cpm/μg.
Assay 2 - DHA-4-^{14}C, 850 μg., specific activity, 125 cpm/μg. and pregnenolone, 880 μg.
Assay 3 - DHA-4-^{14}C, 850 μg., specific activity, 125 cpm/μg. and 17α-hydroxypregnenolone, 880 μg.
Incubation Time: 90 minutes. Gas-phase: air.

Isolated steroids	Assay 1			Assay 2 (pregnenolone)			Assay 3 (17α-hydroxypregnenolone)		
	Tcpm	μg.	Specific activity cpm/μg.	Tcpm	μg.	Specific activity cpm/μg.	Tcpm	μg.	Specific activity cpm/μg.
Androstenedione	21,790	183	119	14,496	128	113	23,700	206	115
DHA	69,840	525	133	76,880	600	128	66,794	526	127
7-keto-DHA	2,964	23	129	4,214	32	130	3,200	25	128
Peak IV	1,686	-	-	1,578	-	-	1,600	-	-
Peak V	3,132	-	-	2,894	-	-	3,744	-	-

The specific activities of the DHA isolated from each of the flasks is similar, so that none of the added pregnenolone or 17α-hydroxypregnenolone was converted to DHA by this normal gland. The specific activities of the isolated androstenedione and 7-keto-DHA are all within the same range as the isolated substrates except perhaps for the androstenedione of the pregnenolone flask which is 12% lower than the corresponding DHA-4-^{14}C. Consequently, some androstenedione might have been formed from the added pregnenolone, although this decrease in specific activity is probably not significant.

Thus, in this normal gland (Gland 51), no inhibition was exerted by 17α-hydroxypregnenolone upon the conversion of DHA to androstenedione, but pregnenolone, as in the hyperplastic gland (Gland 50), led to a significant decrease in the conversion of DHA to androstenedione.

The bovine adrenal gland (bovine gland 2) was studied to ascertain whether pregnenolone and 17α-hydroxypregnenolone exerted similar effects upon the conversion of DHA-4-^{14}C to androstenedione as in human adrenal glands. Similar amounts of pregnenolone and 17α-hydroxypregnenolone relative to DHA were added to the flasks as were present in the incubations of human glands 50 and 51. The results are tabulated in Table LVII.

Bovine adrenal glands do not produce, in vitro from DHA,

TABLE LVII

UTILIZATION OF DHA-4-^{14}C BY A BOVINE ADRENAL GLAND IN THE PRESENCE OF PREGNENOLONE AND 17α-HYDROXYPREGNENOLONE

(BOVINE GLAND 2)

Medium: KRBG with added 0.01M nicotinamide
Tissue: 25 ml. of 10% (W/V) homogenate
Additions: NAD, 60 mg. Final Volume: 27 ml.
Substrate: See below
Incubation Time: 90 minutes. Gas-phase: 95% oxygen and 5% carbon dioxide.

	Isolated Steroids		
	Androstenedione		Other UV absorbing steroids *
Substrates	Tcpm	Specific activity cpm./μg.	Tcpm
DHA-4-^{14}C, 1,700 μg. specific activity, 125 cpm/μg.	180,360	133	5,328
DHA-4-^{14}C, 1,700 μg., specific activity, 125 cpm/μg. with added 2,200 μg. pregnenolone	176,280	113	5,872
DHA-4-^{14}C, 1,700 μg., specific activity, 125 cpm/μg. with added 35.2 mg. pregnenolone	150,264	103	6,682
DHA-4-^{14}C, 1,700 μg., specific activity, 125 cpm/μg. with added 2,200 μg. 17α-hydroxy-pregnenolone	176,536	125	8,100
DHA-4-^{14}C, 1,700 μg., specific activity, 125 cpm/μg., with added 35.2 mg. 17α-hydroxy-pregnenolone	178,416	102	8,200

* 11β-hydroxyandrostenedione, adrenosterone and testosterone identified in this fraction from every incubation.

appreciable amounts of 7-keto-DHA. The formation of androstenedione, 11β-hydroxyandrostenedione, adrenosterone and testosterone was found in the present study. The recoveries of the total cpm., added as substrate, was between 86% and 87% for all the flasks with the exception of that in which 35.2 mg. of pregnenolone was added. Here the recovery was only 74%.

If the recovery in this incubation is corrected for losses, then similar amounts of radio-activity were incorporated into androstenedione from DHA in all the flasks, no inhibition of the conversion of DHA occurring with either approximately equimolar or very high amounts of the C_{21} Δ_5-3β-hydroxysteroids. The total cpm. incorporated into the 11β-hydroxyandrostenedione, adrenosterone and testosterone fraction tended to rise in the flasks containing either pregnenolone or 17α-hydroxypregnenolone, especially the latter, so that there was no inhibition in their formation. No DHA-4-^{14}C was recovered from any of the flasks.

The specific activities of the recovered androstenedione in the control flask and that with 2,200 μg. of 17α-hydroxypregnenolone are the same. However, some androstenedione may be formed from pregnenolone and 17α-hydroxypregnenolone, more especially when they are present in high amounts, judged by the lower androstenedione specific activities from these flasks.

Thus, in bovine adrenal glands, no inhibition of the conversion

of DHA to androstenedione occurs in the presence of equimolar or very high amounts of pregnenolone or 17α-hydroxypregnenolone. This is in agreement with the work of Kowal and his colleagues (1964b) who noted no inhibition of the conversion of DHA to androstenedione by either of these C_{21} Δ_5-3β-hydroxysteroids in acetone powder preparations of bovine adrenal glands.

In bovine adrenal tissue, different Δ_5-3βHSD systems exist for the Δ_5-pregnenes and Δ_5-androstenes, while in human adrenal glands, different enzymes are present for DHA and 17α-hydroxypregnenolone. The inhibition of the conversion of DHA to androstenedione exerted by pregnenolone may be due to either interference or competition with some enzyme involved, in which case they may show a common Δ_5-3βHSD in part or whole, or a direct inhibiting effect. However, the degree of inhibition noted is unlikely to be due to a direct effect as the large amounts of pregnenolone added should have led to almost inhibition under those circumstances.

UTILIZATION OF DHA-4-^{14}C BY A HUMAN BENIGN VIRILISING ADRENAL TUMOUR (GLAND 25)

A further adrenal tumour, probably benign, associated with virilism in a young male child, aged 15 years, has been studied. The incubations were planned to assay the Δ_5-3βHSD system for DHA and to observe if 7-keto-DHA was formed by the tumour and whether a similar pathway was present as was observed in normal and hyperplastic glands.

METHODS

The Δ_5-3βHSD system for DHA was assayed in duplicate by a modification of the method of Rubin et al. (1961) described previously (p.128) by incubating 1.6 ml. of a 20% (W/V) homogenate with DHA. Further assays were performed in triplicate using DHA-4-^{14}C as substrate. To one of these flasks, 732 μg. of unlabelled androstenedione were added as a trap. The incubates were extracted and chromatographed initially upon a 1.5 g. silica gel column and then the combined column fractions were re-chromatographed on a 7.5 g. alumina column.

Further incubations were performed using KRPG as the medium and utilizing 7.5 ml. of a 10% (W/V) homogenate. DHA-4-^{14}C was the substrate in two flasks, to one of which 732 μg. of androstenedione was added as a trap. Androstenedione-4-^{14}C served

as the substrate in the third flask. All the samples were incubated for 90 minutes and following extraction, they were chromatographed and over-run in a Bush B_1 system for 8 hours. The overflows, which would contain steroids of the polarity of DHA, androstenedione, adrenosterone, testosterone and 11β-hydroxy-androstenedione, were submitted to alumina column chromatography. The 7-keto-DHA zone from the Bush B_1 paper chromatogram was eluted and chromatographed on a separate alumina column.

The column fractions corresponding to 11β-hydroxyandrostenedione, testosterone and adrenosterone were rechromatographed using the Bush A, B_3 and B_1 systems with appropriate carrier steroid added.

The procedures to isolate any labelled DHA formed from androstenedione-4-^{14}C by the tumour were performed as described on page 207.

RESULTS AND DISCUSSION

The results of the studies on the utilization of DHA in which the total U.V. absorbing steroids were measured following silica gel column chromatography have been presented previously (Gland 25, Table XXVIII). The mean value of 0.90 µg. U.V. absorbing steroid formed per minute per mg. nitrogen is not a true measure of the Δ_5-3βHSD activity of the tissue, due to the formation of both androstenedione and 7-keto-DHA by the tumour. Subsequent alumina

chromatography showed that the proportion of androstenedione to the total U.V. absorbing steroids formed ranged from 15% to 76% (Table LVIII). The mean activity of the Δ_5-3βHSD system for DHA is 0.35 µg. androstenedione formed per minute per mg. nitrogen, while that for the enzyme system(s) leading to 7-keto-DHA is 0.55 µg. per minute per mg. nitrogen. No equivalent assays have been performed with normal adrenal tissue in which the androstenedione and 7-keto-DHA have been separated so that no direct comparison of the activity of these enzyme systems can be made with that of normal tissue. However, the sum of the activities of both enzyme systems (0.9 µg. per minute per mg. nitrogen) is less than that found for their sum in normal and hyperplastic tissues as measured by the silica gel column technique (between 1.83 and 1.94 µg. per minute per mg. nitrogen, Table XXIII). This may indicate a relative deficiency of both systems in this tumour.

The variation in the proportions of the two steroids formed by the tumour (Table LVIII) is similar to that found in other adrenal gland incubations, yet the sum of the steroids formed is similar in amount in each assay within the incubation.

In Table LIX, the effect of an androstenedione trap upon the Δ_5-3βHSD system for DHA is seen. An apparent enhancement of the reaction would seem to have occurred, indicated by the increase in total cpm. incorporated into androstenedione in the presence

TABLE LVIII

UTILIZATION OF DHA AND DHA-4-^{14}C BY A HUMAN VIRILIZING ADRENAL TUMOUR (GLAND 25)

Medium: 0.154 M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 1.6 ml. of 20% (W/V) homogenate
Additions: 6 mg. NAD. Final Volume: 1.8 ml.
Substrate: 1) DHA, 200 µg.
2) DHA-4-^{14}C, 130 µg., specific activity 479 cpm/µg.
Incubation Time: 30 minutes. Gas-phase: air.

	Isolated Steroids		
	Androstenedione	7-keto-DHA	
Substrate	µg. formed	µg. formed	Total µg. formed from DHA
DHA	11	61	72
DHA	51	16	67
DHA-4-^{14}C	17	50	67

Mean µg. androstenedione formed per minute per mg. nitrogen - 0.35

Mean µg. 7-keto-DHA formed per minute per mg. nitrogen - 0.55

TABLE LIX

UTILIZATION OF DHA-4-^{14}C AND ANDROSTENEDIONE-4-^{14}C BY A HUMAN VIRILISING ADRENAL TUMOUR (GLAND 25)

Medium: 0.154 M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 1.6 ml. of a 20% (W/V) homogenate
Additions: NAD, 6 mg. Final Volume: 1.8 ml.
Substrate: 1) DHA-4-^{14}C, 130 µg., specific activity, 479 cpm/µg.
2) DHA-4-^{14}C, 130 µg., specific activity, 479 cpm/µg. with added unlabelled androstenedione, 732 µg.
Incubation Time: 30 minutes. Gas-phase: air.

Column peak	Isolated steroids	Substrate: DHA-4-^{14}C			Substrate: DHA-4-^{14}C with added androstenedione as a trap		
		Tcpm	µg.	Specific activity cpm/µg.	Tcpm	µg.	Specific activity Cpm/µg.
I	Androstenedione	6,216	14.2	436	15,186	550.5	27.6
II	DHA	13,828	31	446	6,846	15.8	433
Shoulder of II	*	702	-	-	618	-	-
III	7-keto-DHA	17,382	41	424	7,866	23.3	337
IV	-	2,376	-	-	612	-	-
V	-	1,656	-	-	588	-	-

* Substances of the polarity of 11β-hydroxyandrostenedione, adrenosterone and testosterone

of the trap when compared with the control flask with DHA-4-^{14}C alone. However, in view of the variability in androstenedione formed as noted in the other assays (Table LVIII), the androstenedione formed from DHA-4-^{14}C may represent only slight inhibition in the presence of the trap. From the ratio of the specific activities of the isolated DHA and 7-keto-DHA (424 cpm/µg.: 337 cpm/µg., Table LIX) it is possible to calculate that 79% of the 7-keto-DHA arose directly from DHA-4-^{14}C, the remainder coming from the androstenedione trap. The ratio found in a hyperplastic gland for this product was 69% (Gland 48, Table XLVI).

Greater amounts of both androstenedione and 7-keto-DHA were formed from DHA-4-^{14}C when larger amounts of the tumour tissue were incubated for a longer period of time (Table LX). 74 µg. of androstenedione were formed from DHA-4-^{14}C by the control, while only 53 µg. were formed from DHA-4-^{14}C in the presence of the androstenedione trap. This is calculated from the total cpm. incorporated into androstenedione related to the specific activity of the isolated substrate DHA-4-^{14}C (24,323 ÷ 460 = 59 µg.).

As 90 µg. of 7-keto-DHA were formed in the control flask and 103 µg. were formed from DHA-4-^{14}C in the flask with added androstenedione (Calculated from the total cpm. incorporated into 7-keto-DHA related to the specific activity of the isolated substrate

TABLE LX

UTILIZATION OF DHA-4-^{14}C AND ANDROSTENEDIONE-4-^{14}C BY A HUMAN VIRILISING ADRENAL TUMOUR (GLAND 25)

Medium: KRPG with added 0.01M nicotinamide
Tissue: 7.5 ml. of 10% (W/V) homogenate
Additions: NAD, 30 mg. Final Volume: 8.5 ml.
Substrate: 1) DHA-4-^{14}C, 260 µg., specific activity, 479 cpm/µg.
2) DHA-4-^{14}C, 260 µg., specific activity, 479 cpm/µg., with added androstenedione, 732 µg.
3) Androstenedione-4-^{14}C, 500 µg., specific activity, 435 cpm/µg.
Incubation Time: 90 minutes. Gas-phase: air.

Column peak	Isolated steroids	Substrate: DHA-4-^{14}C			Substrate: DHA-4-^{14}C with added androstenedione as a trap			Substrate: Androstenedione-4-^{14}C		
		Tcpm	µg.	Specific activity cpm/µg.	Tcpm	µg.	Specific activity cpm/µg.	Tcpm	µg.	Specific activity cpm/µg.
I	Androstenedione	35,208	74	473	24,323	553	44	95,650	220	457
II	DHA	1,877	3.8	473	1,668	3.6	460	5,584	-	-
Shoulder of II	*	7,159	-	-	8,611	-	-	10,788	-	-
III	7-keto-DHA	42,667	90	474	47,262	148	320	10,756	24.4	440

* Substances of the polarity of 11β-hydroxyandrostenedione, adrenosterone and testosterone

DHA-4-^{14}C, 47,262 ÷ 460 = 103 μg.)., some decrease in the utilization of DHA by the Δ_5-3βHSD system would appear to have occurred, when compared with the rates of formation of the two substances indicated in Table LX.

70% of the 7-keto-DHA formed in the presence of the androstenedione trap is derived from DHA-4-^{14}C. This is calculated from the ratio of the specific activities of DHA and 7-keto-DHA isolated (460 cpm/μg.: 320 cpm/μg., Table LX). This compares well with the 79% formed from DHA-4-^{14}C by the other incubation of the tumour (Table LIX) and by the 69% formed from DHA-4-^{14}C by an hyperplastic gland (Gland 48, Table XLVI).

Additional proof that 7-keto-DHA could be formed by this tumour from androstenedione as in normal and hyperplastic human adrenal glands was shown by the finding of its formation from an incubation in which androstenedione-4-^{14}C was the sole substrate, 24 μg. of labelled 7-keto-DHA being isolated (Table LX).

Table LXI summarises the activities of the two enzyme systems studied in this tumour. Their activities show a marked decrease with increasing amounts of tissue and time of incubation. Although no normal or hyperplastic human gland has been incubated under exactly comparable conditions, a comparison can be made with Gland 48 (Table XLVIII). Even taking into account the decrease in rate of DHA utilization with increasing tissue amounts, the

TABLE LXI

UTILIZATION OF DHA-4-^{14}C AND ANDROSTENEDIONE-4-^{14}C

BY A HUMAN VIRILISING ADRENAL TUMOUR (GLAND 25)

, The data recorded in this table are derived from Tables LIX and LX

Tissue concentration mg.	Incubation time min.	Substrate	Isolated Steroids			
			Androstenedione		7-keto-DHA	
			µg. formed	µg. formed per minute per mg. nitrogen	µg. formed	µg. formed per minute per mg. nitrogen
320	30	DHA-4-^{14}C	17	0.222	50	0.651
320	30	DHA-4-^{14}C with added androstenedione	47	0.613	31.5*	0.410
					7.0≠	0.91
750	90	DHA-4-^{14}C	74	0.078	90	0.094
750	90	DHA-4-^{14}C with added androstenedione	53	0.056	10.3*	0.108
					45≠	0.047
750	90	Androstenedione-4-^{14}C	-	-	24	0.025

* µg. 7-keto-DHA formed from DHA-4-^{14}C

≠ µg. 7-keto-DHA formed from androstenedione (using calculations shown on pp. 237 & 239)

rate found in this tumour is much lower, possibly indicating a relative deficiency of the Δ_5-3βHSD system for DHA and the system(s) leading to 7-keto-DHA formation from DHA.

Peak II from the alumina column of the androstenedione-4-^{14}C incubation was thought to represent DHA (Table LX). Following the addition of authentic carrier DHA to the column fraction and its acetylation, the radio-activity separated from carrier DHA on a Bush A chromatogram.

The radio-activity corresponding to the mobility of 11β-hydroxyandrostenedione, testosterone and adrenosterone, eluted from the alumina column (Table LX), was submitted to paper chromatography after the addition of 100 μg. of authentic appropriate carrier steroids. The zones corresponding to either adrenosterone or testosterone contained no radio-activity. 11β-hydroxyandrostenedione was formed from both DHA-4-^{14}C and androstenedione-4-^{14}C (Table LXII). Since the total cpm. found in the 11β-hydroxyandrostenedione zone after the addition of carrier, when DHA-4-^{14}C was the substrate in the presence of androstenedione as a trap, were greater than the equivalent 11β-hydroxyandrostenedione zone isolated when either DHA-4-^{14}C or androstenedione-4-^{14}C were the sole substrates, it is possible that the traces of 11β-hydroxyandrostenedione may be formed mainly from DHA directly, not by 11β-hydroxylation of the formed androstenedione. Similar

TABLE LXII

UTILIZATION OF DHA-4-^{14}C AND ANDROSTENEDIONE-4-^{14}C BY A HUMAN VIRILISING ADRENAL TUMOUR (GLAND 25)

The formation of 11β-hydroxyandrostenedione.

100 μg. of authentic carrier steroid added as carrier

Substrate	Tcpm isolated after chromatography	Specific Activities (cpm/μg.) after paper chromatography		
		Bush A	Bush B_1	Bush B_3
DHA-4-^{14}C	382	4.4	4.5	4.2
DHA-4-^{14}C with added unlabelled androstenedione	584	7.6	7.0	7.2
Androstenedione-4-^{14}C	156	2.5	2.2	2.3

findings were noted in a normal and an hyperplastic human adrenal gland (Glands 47 & 48, Table XLIX).

The lack of testosterone formation, in even trace amounts, in any of the incubations of the tumour cited, is puzzling considering that the lesion was of the virilising type. However, if a relative deficiency of the Δ_5-3βHSD system for DHA utilization was present, it may be that the large amounts of secreted DHA could itself have led to the virilism.

A STUDY OF THE Δ_5-3βHSD SYSTEMS IN AN ADRENOCORTICAL TUMOUR OF THE MOUSE

Female mice of the CE strain are known to develop spontaneous adrenocortical tumours when gonadectomised shortly after birth. No reports of their steroid biosynthetic activity *in vivo* or *in vitro* appear to have been recorded in the literature.

The principal reason for examining the biosynthetic pathways in these tumours was to determine whether they possessed Δ_5-3βHSD activity, not only for DHA but also pregnenolone and 17α-hydroxy-pregnenolone.

Recently Fukushima and Gallagher (1963) studied three "non-functioning" human adrenal tumours and found evidence in two of the lesions, from urinary studies, of a deficiency of the Δ_5-3βHSD acting upon pregnenolone. In the other patient, there was no evidence of abnormal steroid function. Thus, although some tumours cause no endocrine upsets, this may be due not to their being "non-functioning", but to the absence of the necessary enzymes required for active steroid hormone synthesis, so that they are able to produce only non-hormonally active steroids.

Dr. John Anderson and Miss Maureen McIntyre, working in the University Department of Pathology at Glasgow Royal Infirmary, were able to show that successful tissue culture of these CE strain adrenal tumours was possible. Incubations were carried out after

20 days culture to determine whether the viable cells still possessed the same steroid biosynthetic enzymes as could be demonstrated in vitro immediately after killing the mice.

METHODS

1. Incubation of CE Mouse Adrenal Tumour Tissue Homogenates.

Five complete tumours were homogenised in a medium consisting of equal volumes of 0.154M sodium chloride and 0.01M phosphate buffer to give a 10% (W/V) final tissue concentration. 10 ml. of this preparation were added to each of four flasks, containing 60 mg. of NAD in a final volume of 12 ml. Different substrates were added to each flask: 500 µg. of pregnenolone - 16T (specific activity, 4.4×10^5 cpm/µg.), 500 µg. of 17α-hydroxypregnenolone-7T (specific activity 1.5×10^5 cpm/µg.) or 520 µg. of DHA-4-^{14}C (specific activity, 479 cpm/µg.). A fourth flask was prepared using DHA-4-^{14}C as substrate as before, but containing 1,474 µg. of unlabelled androstenedione as a trap. The incubates were shaken at 37°C in air for 90 minutes. At the end of this time, after dilution with 2 volumes of water, they were extracted three times with 5 volumes of benzene:chloroform (6:1) and the pooled extracts of each incubate taken to dryness.

The extracts from flasks incubated with pregnenolone-16T and 17α-hydroxypregnenolone-7T were submitted to paper chromatography.

After tentative identification of progesterone, 17α-hydroxyprogesterone, deoxycorticosterone, 11-deoxycortisol, and cortisol appropriate carrier steroids were added to the eluted zones and the material taken to constant specific activity using the paper chromatographic systems of Bush and of Savard and in some cases silica gel chromatography as previously described. Derivatives of all of the eluted steroids with the exception of progesterone were made as subsequently shown. The extracts from the DHA-4-^{14}C incubations were submitted directly to alumina column chromatography as described previously.

2. Analysis of Tissue Culture Medium.

After 20 days of tissue culture, the medium, Glaxo 199 (pH 7.4) from two cultures, was replaced with 5 ml. of a fresh solution containing 12 µg. of progesterone-4-^{14}C (specific activity, 9.15×10^4 cpm/µg.) dissolved in 0.01 ml. of propylene glycol. The flasks were replaced in the incubator and incubated without shaking at 37°C for 24 hours in an atmosphere of 5% carbon dioxide in air (Anderson & McIntyre, personal communication).

The cells and medium from one flask and the medium only from the other flask (these cells being kept for autoradiography), were extracted, after dilution with water, three times with 3 volumes of benzene:chloroform (6:1). The pooled dried extracts were submitted to paper chromatography. After preliminary

identification of the steroids, suitable carrier steroids were added and the material taken to constant specific activity using the paper techniques of Bush and of Savard and silica gel chromatography for 17α-hydroxyprogesterone. Derivatives of all of the steroids were formed as will be described.

RESULTS AND DISCUSSION

The steroids formed by the tumour from 17α-hydroxypregnenolone are shown in Table LXIII. Each of these steroids was taken to a constant specific activity (cpm/µg.) by the procedures indicated. The formation of 17α-hydroxyprogesterone, 11-deoxycortisol and cortisol could only be expected to occur if a Δ_5-3βHSD system is present capable of acting upon 17α-hydroxypregnenolone.

Mature mouse adrenal glands appear either to be unable to form 17-hydroxylated steroids or to form them only in trace amounts (Hofman, 1956; Bloch & Cohen, 1960; Varon & Touchstone, 1964). Thus a Δ_5-3βHSD system is present in the tumour capable of acting upon C_{21} Δ_5-3β-hydroxysteroids possessing a 17α-hydroxyl group.

Labelled progesterone, deoxycorticosterone, 17α-hydroxyprogesterone and cortisol were isolated from the tumour incubation with Δ_5-pregnenolone as substrate (Table LXIV). Each was taken to constant specific activity after the addition of carrier by the procedures indicated. Thus the presence of a Δ_5-3βHSD capable

TABLE LXIII

UTILIZATION OF 17α-HYDROXYPREGNENOLONE BY MOUSE ADRENAL TUMOURS

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 10 ml. of 10% (W/V) homogenate
Additions: NAD, 60 mg. Final Volume: 12 ml.
Substrate: 17α-hydroxypregnenolone-7αT, 500 µg., specific activity, 1.5×10^5 cpm/µg.
Incubation Time: 90 minutes. Gas-phase: air.

Isolated steroids	µg. carrier steroid added*	Procedures	Steroid Derivatives	Chromatography		Specific activity cpm./µg.
				Paper	Column	
17α-hydroxy-progesterone	250	-	-	Bush B_3	-	956
		-	-	Bush A	-	710
		-	-	-	Silica Gel	603
		Oxidation	Androstenedione	Bush A	-	626
11-deoxycortisol (compd. S)	100	-	-	Bush B_3	-	589
		Acetylation	S-acetate	Bush A	-	407
		Saponification	Compd. S	Bush B_1	-	388
Cortisol (compd. F)	100	-	-	Bush B_5	-	488
		Acetylation	F-acetate	Bush B_1	-	111
		Saponification	Compd. F	Bush B_5	-	107

* Carrier steroid added after preliminary identification using paper chromatography

TABLE LXIV

UTILIZATION OF PREGNENOLONE BY MOUSE ADRENAL TUMOURS

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 10 ml. of 10% (W/V) homogenate
Additions: NAD, 60 mg. Final Volume: 12 ml.
Substrate: Pregnenolone-16T, 500 µg., specific activity 3.52×10^5 cpm/µg.
Incubation Time: 90 minutes. Gas-phase: air.

Isolated steroids	µg. carrier steroid added*	Procedure	Steroid Derivatives	Chromatography		Specific activity cpm/µg.
				Paper	Column	
Progesterone	250	-	-	Ligroin - propylene glycol	-	172
		-	-	-	Silica Gel	155
		-	-	Bush A	-	140
Deoxycorticosterone (DOC)	100	-	-	Ligroin - propylene glycol	-	270
		Acetylation	DOC acetate	Ligroin - propylene glycol	-	268
		Saponification	DOC	Bush A	-	272
17α-hydroxy progesterone	250	-	-	BUsh A	-	187
		-	-	-	Silica Gel	189
		Oxidation	Androstenedione	Bush A	-	183
Cortisol (compd. F)		-	-	Bush B_5	-	223
		Acetylation	F acetate	Bush B_1	-	228
		Saponification	Compd. F	Bush B_5	-	225

* Carrier steroid added after preliminary identification using paper chromatography

of acting upon Δ_5-pregnenolone has also been shown. Surprisingly, 17-hydroxylated Δ_4-3-ketosteroids (17α-hydroxyprogesterone and cortisol) were also found in this extract. Whether these steroids were formed via the 17α-hydroxylation of progesterone or of pregnenolone is not known. If the pathway is similar to that occurring in human adrenal glands, then 17α-hydroxypregnenolone may be the more likely intermediate (Eichorn & Hechter, 1957; Berliner et al., 1958; Eichorn & Hechter, 1959; Mulrow & Cohn, 1961; Lipsett & Hökfelt, 1961; Weliky & Engel, 1962).

17α-hydroxylation has been shown to occur in incubations of adrenal glands from immature mice and its activity was found to diminish with the development of sexual maturity (Varon et al., 1963; Varon & Touchstone, 1964). The present tumours were derived from female mice which had been oophorectomized shortly after birth.

In the extract derived from the incubation of pregnenolone, no radio-activity was demonstrable in corticosterone added as carrier. Several other steroids were formed from pregnenolone and 17α-hydroxypregnenolone, but only the major steroid products were isolated and identified.

DHA-4-^{14}C was metabolised by the tumour in a manner similar to that in human adrenal glands (Table LXV). Not only androstenedione and 7-keto-DHA were formed, but also the radio-active

TABLE LXV

UTILIZATION OF DHA BY MOUSE ADRENAL TUMOURS

Medium: 0.154M sodium chloride and 0.1M phosphate buffer (1:1)
Tissue: 10 ml. of 10% (W/V) homogenate
Additions: NAD, 60 mg. Final Volume: 12 ml.
Substrate: DHA-4-^{14}C, 520 µg., specific activity, 479 cpm/µg.
Incubation Time: 90 minutes. Gas-phase: air.

Column peaks	Isolated steroids	Substrate: DHA-4-^{14}C			Substrate: DHA-4-^{14}C with 1474 µg. androstenedione as a trap		
		Tcpm	µg.	Specific activity cpm/µg.	Tcpm	µg.	Specific activity cpm/µg.
I	Androstenedione	134,398	277	485	120,924	1,619	74.7
II	DHA	27,516	59	470	41,225	83	494
III	7-keto-DHA	25,176	54	465	25,794	83	313
IV	-	11,856	-	-	9,120	-	-
V	-	1,820	-	-	1,260	-	-

fractions corresponding to the unknown steroids comprising the alumina column peaks, IV and V, previously described. As before, these steroid zones did not absorb U.V. light.

In the presence of the androstenedione trap, DHA was seen to be converted directly to 7-keto-DHA as in the human tissue to the extent of 63%. This is calculated from the ratio of the specific activities of the isolated 7-keto-DHA and DHA (313 cpm/µg: 494 cpm/µg. = 63.3). This percentage is slightly lower than the 70% and 79% obtained with the human virilising tumour (Gland 25 pp. 237 & 239). From the specific activity of DHA and the total cpm. incorporated into the androstenedione trap, it can be calculated that 245 µg. of DHA-4-^{14}C were transformed directly to androstenedione. This represents a negligable (10%) decrease compared with the control DHA-4-^{14}C incubation,

Thus, these tumours possess the Δ_5-3βHSD systems capable of acting upon pregnenolone, 17α-hydroxypregnenolone and DHA. In addition, since the 17α, 21 and 11β-hydroxylases are all present, the reason why these lesions failed to show any obvious <u>in vivo</u> hormonal function is obscure. It may be related to their vascular supply and drainage as has been suggested for "non-function" in some human adrenal tumours (Dobbie & Symington, 1965).

The compounds identified from the 24 hour incubation of progesterone-4-^{14}C with the tissue culture of the tumour are

shown in Table LXVI. Each was combined with the corresponding carrier steroid after preliminary isolation on paper and constant specific activities were achieved by the methods shown in Table LXVI.

In this instance, deoxycorticosterone and corticosterone were identified as products of progesterone-4-^{14}C. The persistence of the 17α-hydroxylase system was confirmed by the isolation and purification of both 17α-hydroxyprogesterone and cortisol. Thus the biosynthetic capacity of the cells was not altered as a result of the tissue culture procedure.

Progesterone-4-^{14}C of high specific activity was chosen as the substrate in order to obtain identifiable products of reasonable specific acitivty after the addition of carrier. However, on performing the incubation, it was found that the "clones", about 20-40 in all, were responsible for converting an appreciable amount of the added substrate in 24 hours.

Using a histochemical technique, the Δ_5-3βHSD system for DHA was demonstrable in the surviving cells after 20 days of culture (Anderson & McIntyre, personal communication). Presumably the Δ_5-3βHSD system for pregnenolone and for 17α-hydroxypregnenolone was also present, although the demonstration of these systems was not attempted.

This experiment shows the value in relating structure and

TABLE LXVI

UTILIZATION OF PROGESTERONE BY TISSUE CULTURE OF MICE ADRENAL TUMOURS

Medium: Glaxo 199, pH. 7.4
Tissue: Cell culture suspended in 5 ml. medium
Additions: NAD, 6 mg. Final Volume: 5.2 ml.
Substrate: Progesterone-4-^{14}C, 12 µg., specific activity, 9.15 x 10^4 cpm/µg.
Incubation Time: 24 hours. Gas-phase: 5% carbon dioxide in air.

Isolated steroids	µg. carrier steroid added*	Procedures	Steroid derivatives	Chromatography		Specific activity cpm./mg.
				Paper	Column	
17α-hydroxy-progesterone	100	-	-	Bush B_3	-	3,070
		-	-	-	Silica Gel	3,120
		oxidation	Androstenedione	Bush A	-	3,060
Cortisol (compd. F)	100	-	-	Bush B_5	-	4,200
		Acetyl-ation	F. acetate	Bush B_1	-	4,000
		Saponific-ation	Compd. F	Bush B_5	-	3,910
Deoxycortico-sterone (DOC)	100	-	-	Ligroin - propylene glycol	-	46,300
		Acetyl-ation	DOC acetate	Bush A	-	5,850
		Saponific-ation	DOC	Bush B_3	-	5,900
Cortico-sterone (Compd. B)	100	-	-	Bush B_1	-	3,380
		Acetyl-ation	B. acetate	Bush A	-	3,810
		Saponific-ation	Compd. B	Bush B_3	-	4,190

* Carrier steroid added after preliminary identification using paper chromatography

biochemical activity and reveals that these tumours possess steroid biosynthetic enzyme systems similar to those of the human gland. Moreover, this tumour was shown to have the Δ_5-3βHSD systems capable of utilizing DHA, pregnenolone and 17α-hydroxy-pregnenolone. Qualitatively these enzyme systems were still functional after 20 days of tissue culture and were similar in pattern to those found in homogenates of the tumour incubated shortly after death of the animals.

DISCUSSION

The incubation of DHA with human adrenal tissue results in the formation of two principal products, Δ_4-androstene-3,17-dione and Δ_5-androstene-3β-ol-7,17-dione (7-keto-DHA), both of which absorb U.V. light at 240 mμ. Consequently, methods of assay of the Δ_5-3β-hydroxysteroid dehydrogenase (Δ_5-3βHSD) for DHA must, of necessity, differentiate between those two steroids, since, presumably, only androstenedione represents the product of the activity of this enzyme system. Therefore, the technique of assay involving the use of a preparatory silica gel column is of no value. Moreover, methods, in which the formed steroids are measured by direct spectrophotometric comparison of the incubates of human adrenal glands against suitable tissue blanks using similar in vitro incubation conditions and with DHA as substrate, are open to the same criticism (Rubin et al., 1961; Bloch et al., 1962; Rubin et al., 1963).

Separation of androstenedione and 7-keto-DHA is easily achieved using alumina column chromatography. These two steroids are responsible for nearly all of the total U.V. light absorbing steroids measured by the silica gel column technique, as judged by the comparison of the same incubation extracts by silica gel and then alumina chromatography. The trace amounts of 11β-hydroxy-androstenedione, adrenosterone and testosterone, formed in some

incubations, do not contribute appreciably to the total formed steroid from DHA as measured by U.V. light absorption alone.

7-keto-DHA is a biosynthetic product of the human adrenal cortex, the possibility of its being an auto-oxidation product of DHA in vitro having been excluded experimentally (p. 173) and by others (Stárka & Katová, 1962). It was not produced by homogenised bovine adrenal glands in vitro in appreciable amounts, but it was isolated from the mouse adrenocortical tumours incubated with labelled DHA.

Androstenedione, 7-keto-DHA and the increased substrate DHA isolated after incubation all have specific activities which fall within the same range. As there is no significant decrease in the specific activity of the substrate DHA in the incubations, with exception of one virilising tumour (Gland 27, Table LIII), it can be concluded that DHA is not formed from endogenous sources under these in vitro conditions. Consequently, the amounts of androstenedione and 7-keto-DHA measured or found reflect the activities of the Δ_5-3βHSD system and the enzyme system(s) leading to the formation of 7-keto-DHA respectively.

No difference is detected in the activity of either enzyme systems in normal compared with hyperplastic human adrenal glands, although a tendency for greater utilization of DHA appears to occur in hyperplastic tissues. The activity of both enzyme systems

falls with increasing tissue amounts and times of incubation, but still no difference between those two types of adrenal tissue is noted.

The values found in the present work for the activity of the Δ_5-3βHSD system for DHA are much lower than those reported by others (Rubin _et al_., 1961; Bloch _et al_., 1962; Rubin _et al_., 1963). This is due primarily to the separation of the products of DHA utilization, which was not done by the previous workers. They noted a wide variation in the activity of the Δ_5-3βHSD system in two normal adrenal glands (3.9 µg. and 8.2 µg. U.V. absorbing steroid formed per minute per mg. nitrogen). A smaller variation is observed in the present study in both normal and hyperplastic glands. Such variations from gland to gland may be due to differences in the proportions of compact cells, derived from the zona reticularis, present in the homogenate. These cells have been shown histochemically and by _in vitro_ incubation techniques to possess less Δ_5-3βHSD activity than the clear cells of the zona fasciculata (Wattenberg, 1958; Dawson _et al_., 1961; Cavallero & Chiappino, 1962; Grant, 1962). Nothing is known of either the cellular or intracellular distribution of the enzyme system(s) leading to 7-keto-DHA fromation from DHA.

Several factors may affect the accuracy of the present assay methods for these enzyme systems. The reversibility of the Δ_5-3βHSD system _in vitro_ has been reported to occur in isolated

sheep microsomes in the presence of NADH (Ward & Engel, 1964). In one of the present experiments, androstenedione-4-^{14}C was also found to be converted to radio-active DHA in low yield when NAD was omitted from the medium. No labelled DHA was formed from androstenedione-4-^{14}C in several other incubations of hyperplastic and neoplastic adrenal tissue in the presence of NAD. Thus, as NAD was present in all the incubations in which the Δ_5-3βHSD system for DHA was assayed, the results found in the present work appear to be valid.

7-keto-DHA can be derived from androstenedione when the latter is present in high amounts. This is illustrated not only by the decrease in the specific activity of the 7-keto-DHA formed from DHA-4-^{14}C in the presence of an unlabelled androstenedione trap, but by the incubation of androstenedione-4-^{14}C itself. Therefore, androstenedione and 7-keto-DHA presumably are in equilibrium so that the assay procedures used in the present work are still valid for the comparison of one gland with another.

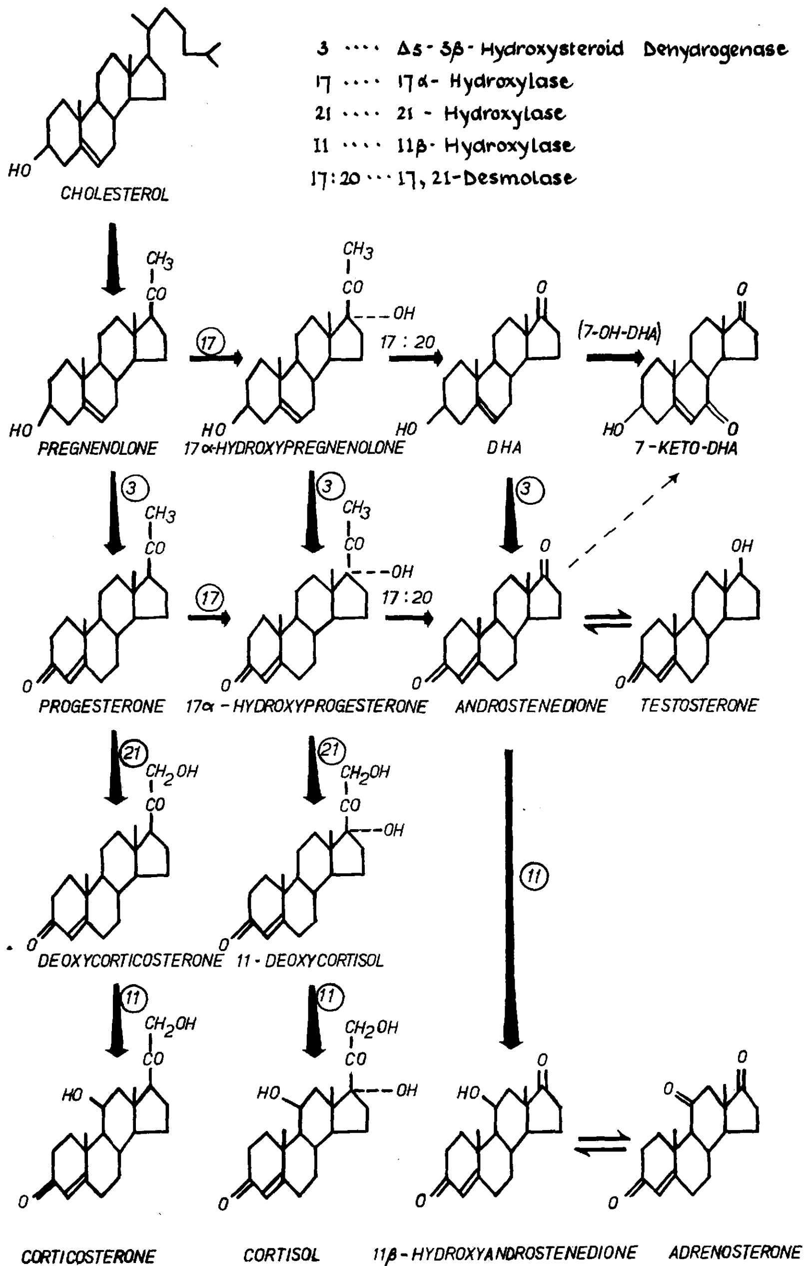
In the present investigation, 7-keto-DHA was found to be formed from two sources. The more important is from DHA directly. 7α-hydroxy-DHA and 7α-hydroxy-oestrone have been shown to be produced by rat liver and bovine adrenal tissues respectively (Šárka & Katová, 1962; Knuppen *et al.*, 1964) while both 7α and 7β-hydroxylation can be effected by micro-organisms (Epstein *et al.*,

1956; Bernstein et al., 1959; Dodson et al., 1959). Thus 7α-hydroxy-DHA may serve as an intermediate in the formation of 7-keto-DHA from DHA and peak IV of the alumina column fractions has the approximate polarity reported for 7α-hydroxy-DHA in the Bush B_5 system (Stárka & Katová, 1962). The other route involves a reversal of the reaction of the 3β-ol-dehydrogenase and Δ_5-3-keto-isomerase systems without apparently involving DHA as an intermediate (Fig. 40), since 7-keto-DHA can be formed from unlabelled androstenedione in the presence of DHA-4-^{14}C without appreciable dilution of the substrate DHA-4-^{14}C (Tables XLV, XLVI & XLVII). This pathway has not been fully investigated but is present in normal, hyperplastic and neoplastic human adrenal tissue.

The rate of formation of U.V. light absorbing steroids from DHA, expressed in μg. per minute per mg. nitrogen, measured by silica gel column chromatography, falls within the same range for normal and hyperplastic adrenal glands. Separation of these steroids into androstenedione and 7-keto-DHA by alumina column chromatography reveals no difference in the average rate of formation of either steroid from DHA in normal or hyperplastic glands (Table XXXVII). However, although the sum of these two steroids formed is similar, the proportion contributed by either one varies inversely, in that when most of the total steroid formed

FIG. 40

MAIN PATHWAYS OF ADRENAL STEROID BIOSYNTHESIS



from DHA is androstenedione, 7-keto-DHA is low. The converse is also true. This suggests the existence of an intermediate compound on the route DHA to androstenedione which can be utilized to form either androstenedione, or presumably _via_ 7-hydroxylation, be converted to 7-keto-DHA and that there is some competition for this intermediate between the enzyme systems responsible for the formation of the two products.

In bovine adrenal gland preparations, androstenedione was shown to inhibit the conversion of DHA to androstenedione _in vitro_ presumably by product inhibition of the 3β-ol-dehydrogenase (Kowal _et al._, 1964b). Thus by inference, the rate of formation of both androstenedione and 7-keto-DHA from DHA would be expected to be decreased in human adrenal tissue in the presence of androstenedione. However, in an hyperplastic human gland and in a virilising tumour, although the formation of androstenedione from DHA-4-^{14}C was in fact found to be decreased in the presence of large amounts of unlabelled androstenedione, 7-keto-DHA was formed in amounts similar to those found from DHA alone (Tables XLVI & LX). These results tend to argue against a common dehydrogenase for which competition exists, but differences exist between the Δ_5-3βHSD system present in human and bovine adrenal glands (see below). The alternative intermediate, for which there is competition, may thus be Δ_5-androstene-3,17-dione.

In the present experiments, no significant radio-activity was detected in the aqueous phase of several incubations after extraction in which phase DHA-sulphate would be expected to remain. DHA-sulphate was found to be formed in vitro by normal (Adams, 1963, 1964; Boström et al., 1964; Killinger & Solomon, 1965) and by neoplastic human adrenal glands (Cohn et al., 1963; Migeon, 1963; Wallace & Lieberman, 1963). DHA-sulphate was detected in vivo in the adrenal and peripheral venous blood and in the urine of cases of adrenal tumours (Baulieu, 1962). Its formation from cholesterol sulphate and pregnenolone sulphate has also been demonstrated in vivo (Calvin et al., 1963; Roberts et al., 1964) although pregnenolone and not pregnenolone sulphate was converted in vitro to DHA-sulphate by normal adrenal tissue (Killinger & Solomon, 1965).

However, the rate of formation of DHA-sulphate from DHA by normal human adrenal glands incubated in vitro was found to be between 21 mμM per g. wet weight per 2 hours and 228 mμM per g. wet weight per hour depending upon the in vitro system (Adams, 1963, 1964; Boström et al., 1964). This corresponds to a rate of 0.01 μg. to 0.1 μg. per minute per mg. nitrogen. Thus the adrenal sulphokinase activity is between 22 and 240 times less active than either the Δ_5-3βHSD system for DHA or of the system(s) for 7-keto-DHA formation. It is thus not surprising that no

significant radio-activity was detected in the aqueous residues where one would expect steroid sulphates of incubates after extraction with the organic solvents.

The Δ_5-3βHSD occupies a key position in the synthesis of adrenal C_{21} and C_{19} Δ_4-3-ketosteroids. Its deficiency in the adrenal cortex has been reported in virilising conditions associated with congenital adrenal hyperplasia (Bongiovanni, 1961; Goldman et al., 1964) and the Stein-Leventhal syndrome (Axelrod et al., 1965) in which disease it is also present in lower activity than normal in the ovary (Axelrod & Goldzieher, 1962).

A deficiency of this enzyme was invoked to explain the high urinary excretion of DHA in patients with adrenal tumours (Rubin & Dorfman, 1957) and by a study of the urinary steroids, such a deficiency was in fact demonstrated in a virilised patient with an adrenal tumour (Lipsett & Wilson, 1962). In the present investigation, an absolute deficiency of a Δ_5-3βHSD system specific for DHA and of the system(s) leading to 7-keto-DHA, were demonstrated in two adrenal tumours (Gland 26 & 27, Table XXVIII). This was confirmed in the latter tumour using DHA-4-^{14}C as substrate. The absence of this enzyme system for DHA has been shown by others using histochemistry (Roversi et al., 1963; Goldman et al., 1964) and by in vitro incubation techniques for pregnenolone conversion (Goldman et al., 1964) in several

virilising adrenal tumours. Not every tumour failed to show Δ_5-3βHSD activity (Goldman et al., 1964). The activity, however, can be less than normal (Gland 25, Tables XXVIII & LXI).

Rubin and her co-workers (1963) reported a relative deficiency, by in vitro techniques, in two carcinomas, one associated with Cushing's syndrome, the other with virilism. It should be noted, however, that since DHA was used as substrate and no attempt was made to distinguish between androstenedione and 7-keto-DHA formation, the result could only be expressed as total U.V. steroids formed per minute per mg. nitrogen. The same criticism can be levelled at the results reported here from the tumour associated with Cushing's syndrome (Gland 24, Table XXVIII).

Thus virilism is not always associated with an absolute deficiency of the Δ_5-3βHSD system but a lowered activity of the system may be seen in some cases. This lowered activity allows the accumulation of DHA and its secretion in excess by such lesions. Although it has been detected as the sulphate ester in adrenal venous blood in such conditions (Baulieu, 1962) and DHA-sulphate has been shown to be metabolically inert in vitro (Kowal et al., 1964b), this does not preclude the sulphate having physiological action as it is freely interconvertible with DHA in vivo (Roberts et al., 1961).

Kowal and his colleagues (1964b) have proposed another suggestion, whereby Δ_5-3βHSD deficiency is not necessarily a prerequisite for the occurrence of virilism, as a result of their finding of an inhibition of DHA utilization by its oxidation products, androstenedione and 11β-hydroxyandrostenedione. They suggested an intracellular feed-back mechanism for the control of DHA utilization, whereby it would accumulate and lead to virilism in the presence of excess amounts of its oxidation products.

The incubation of the virilising adenoma (Gland 27, Tables LIII & LIV) with cholesterol-4-^{14}C as substrate led to the isolation of radio-active Δ_4-3-ketosteroids. This occurred in the absence of any conversion of DHA to androstenedione by aliquots of the same tumour, so that at least two Δ_5-3βHSD systems must exist in the human adrenal cortex, one acting upon DHA and the other, or others, upon the C_{21}-Δ_5-3β-hydroxysteroids. The presence of multiple substrate-specific Δ_5-3βHSD systems was proposed by Weliky and Engel (1962) and confirmed by the in vitro incubation of both human and bovine adrenal gland preparations with DHA and with a variety of hydroxylated C_{21} Δ_5-3β-hydroxysteroids (Berliner et al., 1962; Kowal et al., 1964a; Pasqualini, 1964) and by histochemical methods in the mouse testis (Baillie & Griffiths, 1964).

Bovine adrenal glands have been reported to contain two different Δ_5-3-keto isomerase components of the Δ_5-3βHSD system, one, the Δ_5-pregnene isomerase, acting upon Δ_5-pregnene-3,20-dione and the second, the Δ_5-androstene isomerase, acting upon Δ_5-androstene-3,17-dione (Ewald <u>et al</u>., 1964a; 1964b; 1964c; Krüskemper <u>et al</u>., 1964).

The results obtained in the virilising adenoma (Gland 27, Tables LIII & LIV) also pointed to the existence of at least two Δ_5-3βHSD systems in human adrenal tissue that for DHA being absent in this one case. A study, conducted on two human glands, one normal, the other hyperplastic (Glands 50 & 51, Tables LV & LVI) where the utilization of DHA-4-^{14}C was determined in the presence of pregnenolone and 17α-hydroxypregnenolone, confirmed this view. The formation of androstenedione from DHA in the presence of 17α-hydroxypregnenolone occurred at the same rate as in the incubation with DHA-^{14}C alone even when 17α-hydroxypregnenolone was present in eight times the molar concentration of DHA. The fewer cpm. incorporated into androstenedione in this particular flask, was due to the conversion of unlabelled 17α-hydroxypregnenolone to DHA and by dilution, a decrease in the specific activity of the substrate. Consequently, different Δ_5-3βHSD systems must exist for these two substrates. However, when pregnenolone was present in equimolar or in eight times the molar

concentration of DHA, a significant decrease in the conversion of DHA to androstenedione was found. Therefore, pregnenolone must exert either competition for a component of the Δ_5-3βHSD-isomerase systems for DHA or inhibit these systems directly. The latter seems unlikely as nearly complete inhibition of the utilization of DHA would be expected when pregnenolone is also present in eight times the molar concentration of DHA.

In their study of the Δ_5-3βHSD systems using acetone powder preparations of bovine adrenal glands, Kowal and his associates (1964b) showed that neither pregnenolone nor 17α-hydroxypregnenolone inhibited the conversion of DHA to androstenedione. Using whole homogenates of beef adrenal glands, similar findings were noted in the present work (Table LVII) when equimolar and eight times the molar concentration of both C_{21} Δ_5-3β-hydroxysteroids were incubated with DHA.

It can be concluded that different Δ_5-3βHSD systems exist in bovine glands for C_{21} and C_{19} Δ_5-3β-hydroxysteroids, but that in man, although there are apparently separate systems for DHA and 17α-hydroxypregnenolone, pregnenolone and DHA may share an enzyme system. The finding of deoxycorticosterone and corticosterone formation from cholesterol by the tumour (Gland 27, Table LIV) which lacked the Δ_5-3βHSD system for DHA, points to pregnenolone being utilized by a different Δ_5-3βHSD system.

However, the aetiology of the inhibitory effect of pregnenolone upon DHA utilization must await kinetic studies with purified enzyme systems.

In one normal gland (Table LVI) the formation of 7-keto-DHA from DHA was increased by 39% in the presence of pregnenolone over the control flask, while the formation of androstenedione from DHA was decreased by 43%. This is some evidence for a common enzyme system for the formation of androstenedione and 7-keto-DHA from DHA. This probably is the 3β-ol dehydrogenase since the isomerase most likely is not concerned with 7-keto-DHA formation, and thus the inhibiting effect of pregnenolone may be exerted upon the isomerase component of the Δ_5-3βHSD for DHA.

The occurrence of virilism with high DHA excretion and a low or normal cortisol secretion, due to adrenal tumours, can be explained by assumming two Δ_5-3βHSD systems, the one for DHA being absent or of very low activity, while that for 17α-hydroxypregnenolone or pregnenolone is present, possibly in lower amounts than normal. This could result in a deviation of the biosynthetic pathway to increased DHA formation from either cholesterol or pregnenolone, but still allow near-normal dortisol production. Alternatively, the inhibiting effect of DHA itself upon the conversion of pregnenolone and 17α-hydroxypregnenolone to Δ_4-3-ketosteroids may account for the cortisol output found

in such cases (Kowal *et al.*, 1964b). This latter possibility is unlikely, as the occurrence of Cushing's syndrome and virilism together (i.e., "mixed" Cushing's syndrome) due to adrenal tumours, can be associated with increased DHA excretion (Gallagher, 1958; Lipsett & Wilson, 1962). It is more likely that higher activities of the Δ_5-3βHSD system for the C_{21} Δ_5-3β-hydroxysteroids are present in these tumours by comparison with those only causing virilism and so allow increased formation of cortisol.

The relationship of 7-keto-DHA to the causation of virilism is unknown. The 7-hydroxylation of both androstenedione and 11-deoxycortisol is associated with loss of their physiological effects (Bernstein *et al.*, 1959), so that 7-keto-DHA may represent a metabolic degradation product of DHA. The steric configuration of the Δ_5-7-keto group is similar to that of the physiologically active Δ_4-3-ketosteroids, so that an assessment of its physiological potential would be of great interest.

11β-hydroxyandrostenedione and adrenosterone have been found to be produced on the *in vitro* perfusion of bovine adrenal glands (Bloch *et al.*, 1956) and both DHA and androstenedione have been shown to be precursors of 11β-hydroxyandrostenedione in beef adrenals (Hayano & Dorfman, 1953; Jeanloz *et al.*, 1953; Meyer *et al.*, 1955; Rosenfeld *et al.*, 1955). It was assumed that 11β-hydroxyandrostenedione, adrenosterone and testosterone were

formed from DHA *via* androstenedione. This pathway was found to exist in bovine adrenal tissue in the present work (Table L). These three compounds were found only in trace amounts on *in vitro* incubation of normal and hyperplastic human adrenal glands with DHA-4-^{14}C as substrate and were isolated only after the addition of carrier steroids. In one gland (Gland 49, Table XLIX), with androstenedione-4-^{14}C as substrate, these steroids were detected, but in other incubations (Glands XLVII & XLVIII, Table XLIX), more radio-activity was incorporated into these three steroids from DHA-4-^{14}C than from androstenedione-4-^{14}C, especially with the testosterone fraction. Consequently, an alternative pathway in the formation of these compounds from DHA, not involving androstenedione as an intermediate, may exist in human adrenal glands. Similarly, in one virilising tumour (Gland 25, Table LXII) 11β-hydroxyandrostenedione appeared to be formed from DHA directly. No testosterone formation *in vitro* was demonstrated in this virilising lesion from DHA. That Δ_5-androstene-3β,17β-diol could serve as a precursor of testosterone was shown by the *in vitro* incubation of an human adrenal tumour associated with virilism (Baulieu *et al*., 1963b). This steroid has been isolated from the urine and adrenal venous plasma of patients with virilism (Hirschmann & Hirschmann, 1945; Mason & Kepler, 1945a, 1945b; Hirschmann *et al*., 1960) and is indirect evidence of a decrease

in Δ_5-3βHSD activity for DHA in virilising syndromes.

Thus androstenediol can serve as an intermediate in testosterone formation from DHA, reduction of the 17-ketone group occurring before the action of the Δ_5-3βHSD system. Similarly, 11β-hydroxylation and subsequent 11-oxidation of DHA may take place before the Δ_5-3βHSD system acts, to form 11β-hydroxyandrostenedione and adrenosterone.

11β-hydroxyandrostenedione, in the present investigations was found to be a major product from both DHA and androstenedione only in beef adrenal incubations. It is formed in only trace amounts in human adrenal gland studies from these substrates. While DHA has been reported to inhibit 11β-hydroxylation (Sharma et al., 1963), this cannot be the explanation of the findings in the present incubations for only trace amounts are formed from androstenedione even in the absence of added DHA.

It is difficult to interpret these findings of low rates of formation since 11β-hydroxyandrostenedione has been found to be a major human adrenal steroid secretion as determined in adrenal venous blood (Romanoff et al., 1953; Sweat, 1955; Bush et al., 1956; Grant et al., 1957; Bush & Mahesh, 1959; Lombardo et al., 1959; Short, 1960). A possible explanation may reside in the similarity of the properties of chromatographic mobility and U.V. light absorbtion of 11β-hydroxyandrostenedione and 7-keto-DHA.

However, the latter compound has only been detected as the sulphate in adrenal venous blood (Baulieu, 1962) and 11β-hydroxyandrostenedione isolated as the free steroid and in most instances, characterised beyond doubt. It may be that 11β-hydroxyandrostenedione arises not from either DHA or androstenedione *in vivo*, but by the side chain cleavage of cortisol. In further incubation studies a study of co-factor requirements and the use of DHA-4-^{14}C and androstenedione-4-^{14}C of high specific activity would be necessary before these postulated pathways for the formation of 11β-hydroxyandrostenedione, adrenosterone and testosterone in human adrenal tissue can be elucidated.

Three points of interest arise from the *in vitro* studies of the mouse adrenal tumours occurring spontaneously in gonadectomised female mice of the CE strain. They were found to be capable of forming 7-keto-DHA, from both DHA-4-^{14}C and from androstenedione and in similar amounts from either substrate as was found in the human material studied, This is the first demonstration of the formation of this C_{19} steroid by adrenal tissues of animals other than man.

This study of the Δ_5-3βHSD systems for DHA, pregnenolone and 17α-hydroxypregnenolone indicates that all are present in these tumours.

The presence of 17α-hydroxylation was noted to occur not only in the tumour homogenate studied by *in vitro* incubation, but also in the "clones" from the tumour after 20 days of tissue culture.

Difference of opinion exist as to whether 17α-hydroxylation takes place in the adrenal cortex of mice and rats. It is generally accepted that this 17-hydroxylation system is absent in rats (Peron, 1960; Laplante *et al*., 1964) and mice (Hofman, 1956; Bloch & Cohen, 1960). However, the adrenal tissue of rats with adrenal regeneration hypertension produces 17α-hydroxylated steroids (Brownell *et al*., 1963) and 17α-hydroxylation has been shown to occur in sexually immature mice, this ability being suppressed after sexual maturation (Varon & Touchstone, 1964). The tumours used in the present study were derived from sexually immature mice, which had undergone gonadectomy shortly after birth. *In vitro* incubations with adrenocortical tumours of mice of a different strain showed the presence of 11β and 21-hydroxylase activities and an active 17:20-desmolase (Hofman & Christy, 1961). No appreciable 17α-hydroxylation could be detected, yet these tumours also arose in animals gonadectomised shortly after birth. (Hofman *et al*., 1960).

Consequently, in the adrenal tumours of mice of the CE strain the biosynthetic pathways show a remarkable similarity to those occurring in man (Fig. 40).

SUMMARY

An investigation has been undertaken of the Δ_5-3β-hydroxysteroid dehydrogenase activity in normal and pathological human adrenal tissues, utilizing Δ_5-androstene-3β-ol-17-one (DHA) as substrate.

The principal products of DHA metabolism by the <u>in vitro</u> incubation of human adrenal tissue are Δ_4-androstene-3,17-dione and Δ_5-androstene-3β-ol-7,17-dione (7-keto-DHA), both of which are U.V. light absorbing steroids.

7-keto-DHA has been shown previously to occur in the urine from patients with normal and raised adrenocortical function and to be produced by the <u>in vitro</u> incubation of rat liver, but this is the initial demonstration of its production <u>in vitro</u> by human adrenal glands.

Radiochemical evidence is presented which shows that most of the 7-keto-DHA formed is derived directly from DHA, but that androstenedione can contribute to the 7-keto-DHA pool, if present in large amounts.

Separation of the two principal products of DHA utilization, androstenedione and 7-keto-DHA, by alumina column chromatography gives a measure of the activities of the Δ_5-3β-hydroxysteroid dehydrogenase system and of the enzyme system(s) leading to 7-keto-DHA formation respectively.

The rate of formation of the total U.V. light absorbing steroids (androstenedione and 7-keto-DHA) does not differ in normal and hyperplastic human adrenal glands. The activities of the Δ_5-3β-hydroxysteroid dehydrogenase and the enzyme system(s) for 7-keto-DHA are also found not to differ in those two types of adrenal glands.

One benign and one malignant adrenocortical tumour, both associated with virilism show an absolute deficiency in the conversion of DHA to both androstenedione and 7-keto-DHA.

In the benign tumour, the conversion of cholesterol to U.V. light absorbing steroids (cortisol, corticosterone, deoxycorticosterone and 17α-hydroxyprogesterone) is demonstrated. This is further evidence for the existence of several substrate-specific Δ_5-3β-hydroxysteroid dehydrogenase systems, for one of which (for DHA) an absolute deficiency exists in this tumour.

In vitro experiments using bovine adrenal glands confirmed the existence of different Δ_5-3β-hydroxysteroid dehydrogenase systems for the C_{21} Δ_5-3β-hydroxysteroids, pregnenolone and 17α-hydroxypregnenolone, and for the C_{19} Δ_5-3β-hydroxysteroid, DHA. While there are different systems for 17α-hydroxypregnenolone and DHA in human adrenal glands, the observed inhibitory effect of pregnenolone may be due to either competition for the same enzyme system or to its having a direct inhibitory effect.

The Δ_5-3β-hydroxysteroid dehydrogenase system is found to be a reversible enzymic process converting androstenedione to DHA. The formation of 7-keto-DHA from androstenedione, on the other hand, occurs by a pathway not involving DHA as an intermediate.

The possibility that testosterone, adrenosterone and 11β-hydroxyandrostenedione may be formed by the human adrenal gland by a pathway not necessarily involving androstenedione is raised.

In the study of spontaneous adrenocortical tumours, developing in gonadectomised mice of the CE strain, it is shown that the biosynthetic pathways in the tumours are similar to those of the human adrenal cortex, and include the presence of 17α-hydroxylation.

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