ACTIONS OF CORTICOTROPHIN ON THE HUMAN ADRENAL CORTEX

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BY

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A C K N O W L E D G E M E N T S

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P R E F A C E

PREFACE

The principal object of the studies to be reported was to clarify the nature of the pituitary control of the adrenal cortex in man. The experiments were performed on adrenal glands obtained in a manner previously described by Symington et al., (1956), and their studies were extended to the investigation of the possibility that the adrenal cortex is controlled by more than one pituitary factor, to the study of the effect of corticotrophin on some dehydrogenases of the adrenal cortex, and the work of Griffiths (1960) on effects of corticotrophin on human adrenal cortex *in vitro* was used as the starting point for further experiments. In the course of these studies some observations were made which were subsidiary to the study of the effects of corticotrophin on the human adrenal cortex and these observations are also presented here.

The first part of this thesis is a review of the present knowledge of the principal components of the pituitary adrenal axis, with special reference to man. However, the great bulk of the work has been first performed on animals such as the rat, guinea pig and the ox, and this work is also discussed. Special attention is also given to the nature of the pituitary substance which controls the adrenal cortex, and the possible mechanisms of its action are listed.

The first experimental section deals principally with the varying effects of pituitary preparations on adrenal weight and histological

appearances. The results of an investigation of the normal adrenal weight in operation and post-mortem cases are also present in this section.

The second experimental section deals with the alterations in the activity of the enzymes which can generate the reduced form of nicotinamide-adenine dinucleotide phosphate (NADP) in adrenal cortex of patien who received corticotrophin for four days before the operation. These experiments were also repeated on rats and guinea pigs.

The effects of corticotrophin on the slices of adrenal cortex incubated under artificial conditions are presented in the last section. This was originally an attempt to extend the observations on the importance of NADP linked dehydrogenases in the adrenal cortex reported in the previous section, but has led to results of interest not related to this question.

Several aspects of the work described here have already been reported: (1) "Triphosphopyridine nucleotide-linked dehydrogenases in the Adrenal Cortex in Man". Biochem. J., 78, 4P (1961). This is a summary of a paper read to the Biochemical Society in November 1960. (2) "Effect of 3',5'-AMP on corticosteroid production in vitro by slices of the adrenal cortex of human beings". Nature, 193, 1075, (1962). (3) "Triphosphopyridine nucleotide linked dehydrogenases in the adrenal cortex in Man; the effect of corticotrophin and distribution of enzymes". Acta. Endocrinol., 40, 232, (1962).

GENERAL INTRODUCTION

1. INTRODUCTION

One of the central ideas of endocrinology is the well established principle that the anterior pituitary gland (adenohypophysis) has a controlling effect over several other endocrine glands. This control gives us some justification for referring to the endocrine organs, a widely separated collection of glands without any structural connection, as the "endocrine system". This system has as its prime function the preservation of the organism's internal environment, be it through the acceleration of the metabolic rate, the preservation of water, inorganic ions and glucose, or getting ready for "fight or flight" to prevent external agents interfering with the internal conditions. The second important function of the endocrine system is regulation of the processes associated with growth and reproduction.

The adrenal cortex is an important member of this system. The adrenal gland was first described by Bartholomaeus Eustachius in 1563 when he was studying human anatomy. Almost 300 years elapsed before some function could be attributed to this structure. In 1855, Thomas Addison published a description of the syndrome which now bears his name and which was associated with destruction of the adrenal glands. Brown-Sequard in 1856 showed that extirpation of the animals adrenals is fatal. Later it was established that it is the adrenal cortex which is essential for survival of the animal and not the medulla, and that the adrenal cortex secretes into the blood stream a hormone or

hormones which are necessary for the retention of the sodium ion by the kidney. Adrenal cortex also secretes hormones which influence carbohydrate and protein metabolism and several sex hormones (reviewed by Hechter and Pincus, 1954).

Pituitary control of the adrenal cortex was established in 1927 by Philip Smith, following observations by Evans (1924) that administration of growth promoting substances of pituitary origin to animals causes adrenal hypertrophy. Smith (1927, 1930) evolved an efficient and fairly safe method of pituitary ablation in the rat, and found that this results in marked atrophy of the adrenal cortex, which could be prevented, or the normal condition restored, by intramuscular implantation of fresh rat pituitaries. Other workers (Houssay et al., 1933; Collip et al., 1933) showed that pituitary extracts have the same effect as pituitary implants. The active principle is present in the anterior pituitary (Smith, 1930; McQueen-Williams, 1934), and when released into the general circulation maintains the normal existence of the adrenocortical cell. Marked adrenal hypertrophy can be produced in the intact animal by continued injection of pituitary extracts (Collip et al., 1933; Noble and Collip, 1941). It became clear that a substance is elaborated by the cells of the anterior pituitary gland with a controlling effect on the adrenal cortex and it was called the adreno-cortico-tropic-hormone, abbreviated to ACTH. The official name now is corticotrophin (see p. 5).

"Stress"

Great impetus was given to the study of pituitary-adrenal interrelationships by the concepts proposed by Hans Selye. In his early papers (Selye, 1936, 1937) he does not refer to "stress" by name, but the idea is implicit in the phrase "a syndrome which appears when a severe injury is inflicted upon the organism. This syndrome is independent of the nature of the damaging agent and represents rather a response to damage as such." Such damaging agents include exposure to heat or cold, traumatic injuries, excessive muscular exercise, spinal shock, acute infections and intoxications by various drugs. In general stress can now be taken to mean any stimulus or sequence of stimuli which is harmful or potentially harmful to the animal, and according to Selye, gives rise to a series of changes which he calls the General Adaptation Syndrome. He divides this reaction to stress into three stages. The alarm reaction is seen in the rat six to forty-eight hours after the initial injury, and is characterized by involution of lymphatic organs (thymus, lymph nodes, spleen), loss of muscular tone, formation of acute erosions in the digestive tract, loss of cortical lipid from the adrenal and adrenal hypertrophy.

After a few days a certain resistance is built up against the damaging stimulus. This is the stage of resistance when the organ changes noted above, tend to revert to normal. If the stress is very severe, the animal dies with organ changes seen in the alarm reaction. This is the stage of exhaustion.

The phenomena associated with stress do not occur in the absence of the pituitary gland (Ellison and Burch, 1936; Ingle and Higgins, 1938). This observation and subsequent work has firmly established that the discharge of corticotrophin from the anterior pituitary is an essential link in the series of events initiated by stress. Corticotrophin acting on the adrenal cortex causes the observed changes in the adrenal and promotes increased secretion of the hormones of adrenal cortex. These hormones are responsible for all other effects observed after application of stress. The hypophysectomized animal is extremely sensitive to a variety of non-specific stresses (Baird et al., 1935; Porta, 1935) and the resistance of such animals is increased by administration of adrenocortical extracts (Baird et al., 1935).

These concepts are of great historical importance because not only did they serve to concentrate attention on the pituitary-adrenal relationship, but when their application to human disease became apparent, increasingly greater resources were channelled into adrenal research.

"Corticotrophin"

There is marked inconsistency in the usage of the word corticotrop(h)in. At present the British journals prefer the form corticotrophin, while American publications use the term corticotropin. The relative merits have been the subject of an excellent annotation by Connor (1943), who pointed out that although the term first used by

Collip in 1933 use 'adrenotropic,' the use of -trop- in this way is contrary to its established prior use in biology. It is derived from the Greek work trepein, which means "to turn", and the suffix -tropism was adopted as early as 1854 to indicate a physical movement of an organism or a part of an organism in a specific direction, e.g., heliotropism, phototropism. The etymologically correct meaning of "adrenocorticotropin" is therefore "a substance which is attracted to the adrenal cortex". On the other hand, trophic comes from the Greek trophein, to nourish, and used as a suffix denotes nourishment or nurture. It is argued can also be questioned since corticotrophin may have an effect purely on the function of the adrenal without concomitant nutrient (growth promoting) action, but the term nurture can be considered not to imply growth.

2. THE ADRENAL CORTEX

Since the object of this study was to observe the effects of corticotrophin on the adrenal cortex, the present knowledge of adrenal structure and function is summarized in the following pages. As far as possible, the data refer to the human adrenal gland, but work done on animals often preceded the studies in man and is frequently more detailed. The most important animal studies are therefore also mentioned.

A. Structure.

Adrenocortical tissue is found in all vertebrates but not in the more primitive forms of animal life. In mammals the adrenals are paired organs characteristically situated near the upper pole of each kidney. Each gland consists of two distinct and functionally unrelated parts, cortex and medulla, whose only link is the sharing of the supporting connective tissue and capsule, and a common blood supply. The adrenal medulla produces and stores the catechol amines (adrenaline and noradrenaline) and will not be considered here further.

The characteristic feature of the mammalian adrenal cortex is the arrangement of its cells into three fairly definite concentric zones. This purely morphological subdivision of the cortex has been evident for a very long time. In 1866 Arnold introduced the traditional three zones, glomerulosa, fasciculata and reticularis, on the basis of supporting tissue arrangement. A capsule of connective tissue

surrounds the gland, and is continuous with a fine network of reticulin fibres which surround clusters of epithelial cells. This is the zona glomerulosa. In the zona fasciculata the connective tissue is arranged in a radial form and so the cells of this zone are split up into columns running at right angles to the surface of the gland. In the innermost part of the cortex, zona reticularis, the connective tissue forms a network with large meshes which contain anastomosing cords of adrenal cells (see Fig.10). The small blood vessels in the gland follow the same general pattern as the connective tissue arrangement.

In some species another definite zone can be distinguished. This is a narrow band, only 3 or 4 cells thick, which separates zona glomerulosa from zona fasciculata, and is particularly well seen in rodents. Chester Jones (1957) calls it zona intermedia. This zone is never seen in man.

It has been stated (Neander, 1952) that the variations in the histological appearance of the cortices in different eutherian species are only alterations of a common plan. However, Symington has recently re-emphasised that species differences are important practical considerations in endocrine research (Symington, 1960). One point is that in some species zona glomerulosa is a distinct wide band while in others it is narrow and merges with the zona fasciculata. Also, some species, notably the ruminants and the hamster, have adrenals which are very poor in lipids. In these animals the fasciculata and

reticularis zones cannot be distinguished, but form a uniform zone composed of "compact" cells.

An important feature of the human adrenal cortex is the poor demarcation between certain zones. As mentioned above, zona glomerulosa is sometimes very narrow and always poorly demarcated from the zona fasciculata, which in turn has a more distinct border with the zona reticularis. This border, however, is never straight but follows a sinuous curve resembling the relationship of the epidermis to the dermis. Although the border between the reticularis and medulla is straight and distinct, projections of cortical cells sometimes accompany the blood vessels into the medulla and are seen on cross section island-like among the chromaffin cells. These facts make it difficult to separate the various zones of the human adrenal cortex in order to study their potentialities under in-vitro conditions.

The separation of the zona fasciculata from the zona reticularis is somewhat facilitated, however, by the presence of a bright yellow pigment in the cells of the zona fasciculata. This pigment, probably of carotenoid nature (Behrt, 1904), is absolutely characteristic of the lipid containing cells of the adrenal, and is lost when the gland is depleted of lipid. The fasciculate portion of the human adrenal is therefore golden yellow in colour, and easily distinguished naked eye from the zona reticularis, which is brown.

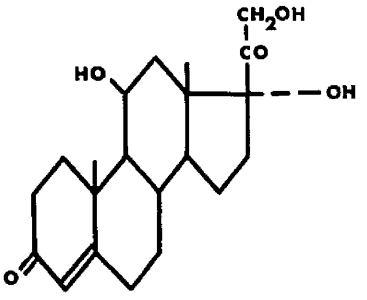
B. Steroid biosynthesis and secretion.

The function of the adrenal

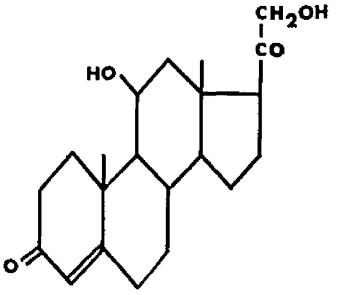
cortex is to produce steroid hormones which are essential for the normal life of the animal. In man the principal hormones identified in adrenal venous blood are cortisol, corticosterone, and aldosterone, and the structural formulae are shown in Fig. 1. Trace amounts of androgens, progesterone and several other C_{21} steroids were also found (reviewed by Grant, 1962). There is also indirect evidence that oestrogens can be produced by the human adrenal cortex (reviewed by Engel, 1962).

The methods used to study the processes of steroid secretion by the adrenal cortex are shown in Fig. 2 in descending order of organisation and biological complexity. The studies performed on urinary and peripheral blood steroids give only indirect information about the adrenal cortex secretion. They constitute an important adjunct to the other studies, but little basic information can be expected from them.

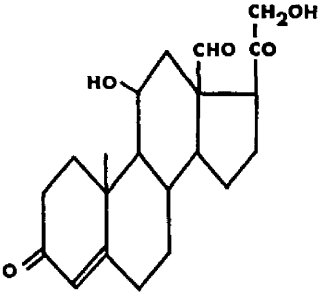
Vogt (1943) was the first to cannulate the adrenal vein of an animal and to study the hormone content of blood so obtained; the human adrenal effluent has been studied by Bush (1953), Romanoff et al., 1953, Sweat (1955), Grant Forrest and Symington (1957), and Short (1960) and the results show that the principal human adrenal hormones are the ones listed above, but pretreatment with corticotrophin preferentially increases secretion of cortisol. The finding of trace amounts of steroids in the adrenal vein in situ is open to the objection that



CORTISOL



CORTICOSTERONE



ALDOSTERONE

Fig. 1. The structural formulae of the principal steroids secreted by the human adrenal cortex.

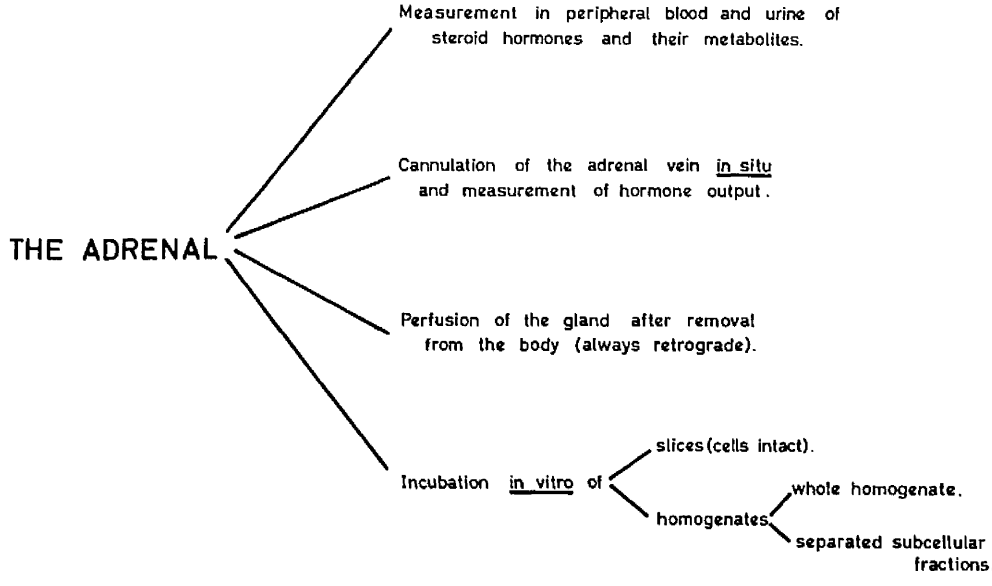


Fig. 2. Methods which have been employed to study steroid production by the mammalian adrenal cortex.

these substances originate in another gland and merely pass through the adrenal in the blood stream.

A great deal of valuable information has been obtained on the cow adrenal by the Worcester group using perfusion techniques (Hechter, 1949; Hechter et al., 1953). While these and all other in vitro techniques can give direct answers to certain questions, they suffer from an inherent limitation that the results obtained are not necessarily of physiological importance under in vivo conditions.

Even simpler in vitro systems have been used. Saffran and colleagues (1952) used adrenal slices incubated in Ringer's solution, and adrenal homogenates and cell fractions are particularly useful for the study of the distribution and nature of the enzymes in the adrenal cortex (e.g., Grant and Brownie, 1955).

The production of steroid hormones involves two separate phenomena, biosynthesis of these substances and their release into the blood stream. It is known that the adrenal gland contains only minute amounts of the steroid hormones (Neher, 1958) so it would appear that release follows closely on biosynthesis. Little is known, however, of the mechanism of hormone release, but it is possible that blood flow in the neighbourhood of a cell can influence the release of steroids by that cell.

The reactions involved in the biosynthesis of adrenocortical steroids can be divided into two groups: those involved in the

synthesis of cholesterol and those causing further transformation of cholesterol (Fig. 3). The first set of reactions is probably common to all cells which can synthesise cholesterol, and most cells have this potential. The original starting material is acetyl coenzyme A, which may be produced *in situ* from glucose and fatty acids. The subsequent reactions require cofactors such as adenosine triphosphate and $NADPH_2$. Some steps are outlined in Fig. 3. Cholesterol, formed as indicated above, is stored mainly in the zona fasciculata cells, partly in the free form and partly esterified. In the human gland 90% of cholesterol is in the esterified form (Fox, 1920). It should be noted that all the cholesterol present in the adrenal need not be produced *in situ*. In the rat most of it arrives to the adrenal from the blood (Morris and Chaikoff, 1959), and in the guinea pig 60% is derived from the blood (Corbin and Chaikoff, 1961).

The reactions between cholesterol and the adrenal steroid hormones have been studied in detail and these reactions, together with the enzymes which promote them are shown in the lower part of Fig. 3. There is no doubt that each of these reactions can occur, but the sequence in which they do occur is the subject of some dispute. Also a controversial question is whether cholesterol is an obligatory intermediate in corticosteroid biosynthesis. While a number of published experiments seem to throw doubt on cholesterol as a direct intermediate in steroid biosynthesis (e.g., Stone and Hechter, 1954; Heard *et al.*,

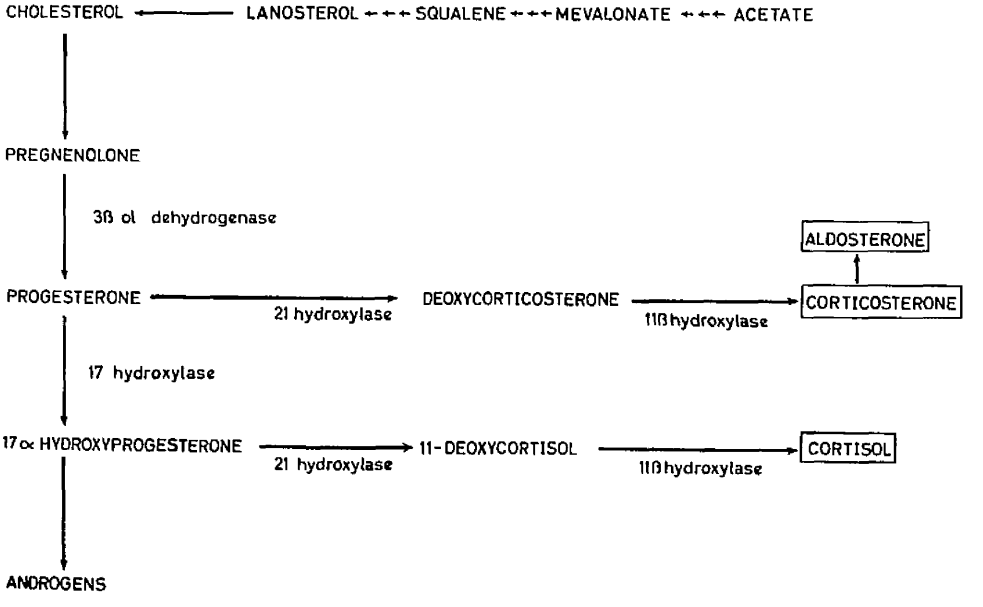


Fig. 3. Principal steps in the biosynthesis of steroid hormones by the adrenal cortex.

1956), such results can be explained in a way which does not exclude cholesterol from the biosynthetic pathway (Grant, 1960). The results of Chaikoff and co-workers referred to above, show that cholesterol must be the major source of adrenal steroid hormones in some species at least.

C. Relation between adrenal structure and function.

It is known that the adrenal cortex of most species can be divided into three seemingly separate zones and that it secretes several hormones with different properties. The question is what hormones are elaborated by the various zones. The original theory of Gottschau ("the escalator theory") proposed in 1883 can now be regarded as disproved. The theory was that the cells originated at the periphery of the gland, migrated inwards and secreted hormones as zona fasciculata, and then degenerated in the zona reticularis. It was replaced by the "zonal theory" which was first proposed by Swann in 1940, and supported by the work of Deane and Greep (1946) who showed that the zona glomerulosa is less dependent on the anterior pituitary than the other zones. This has been fully confirmed, and this zone is now known to produce aldosterone (Ayres et al., 1956; Giroud et al., 1956) and is only under a partial control of the pituitary. The principal trophic factor is "adrenoglomerulotrophin", (Farrell and McIsaac, 1961). The zonal theory, according to Chester Jones (1957), also requires that

the zones fasciculata and reticularis are separate entities; zona fasciculata produces cortisol and corticosterone, while zona reticularis forms adrenal androgens. This view is summarised in Fig. 4. The latter part of this theory is supported by little evidence, while evidence against it is mounting (summarised by Symington, 1968). It has been proposed, by Coffey for the rat (1953), and Symington for the human adrenal cortex (1955), that zona reticularis is the principal site of production of all cortical hormones except aldosterone (Fig. 4). The function of the zona fasciculata is considered in Symington's theory to be merely to store the steroid hormone precursors, which are liberated at times of stress, but it is not excluded that hormones can be contributed to the total output by the zona fasciculata, even in the absence of stress. For instance Griffiths (1960) has shown that under <u>in vitro</u> conditions adrenal slices secrete equal amounts of ultraviolet light absorbing steroids. <u>In vitro</u> findings, however, as stated above, are of questionable physiological significance.

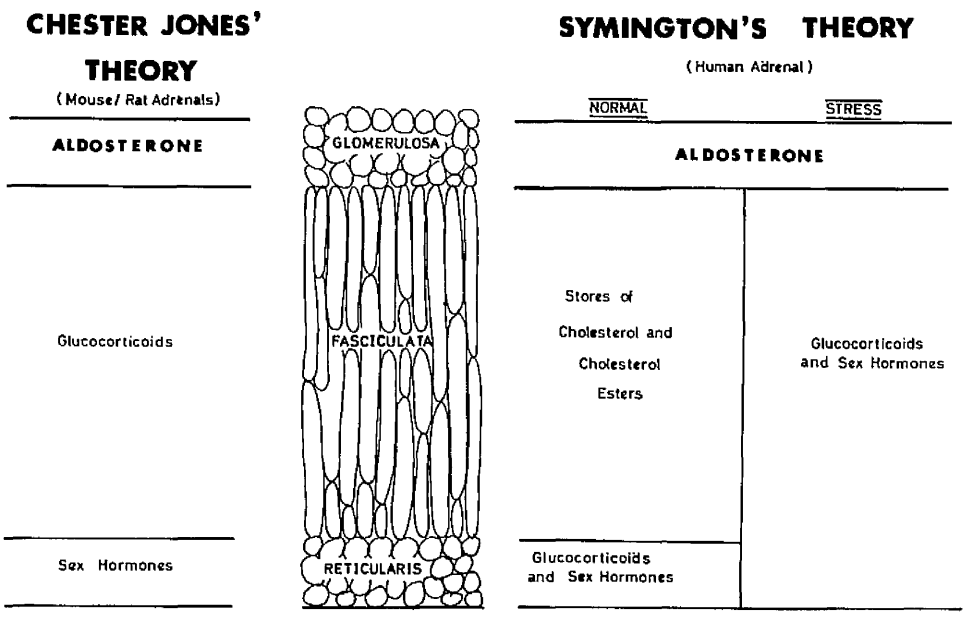


Fig. 4. Two of the recent theories of the relationship between adrenal structure and function.

3. CORTICOTROPHIN

A. Definition of corticotrophin.

When the existence of the pituitary control of adrenal cortex was first demonstrated in 1927, attention was concentrated on the effects of the pituitary extracts on the maintenance of the adrenal weight after hypophysectomy, or the increase in adrenal weight if the adrenal was first allowed to atrophy after hypophysectomy. In order to measure the corticotrophic potency in the extracts, these properties were utilised as biological assays, the two principal methods being the "Adrenal Weight Maintenance" assay and the "Repair" assay (e.g., Simpson et al., 1943). Thus, for twenty years corticotrophin was thought of as a substance secreted by the anterior lobe of the hypophysis to maintain the weight and function of the adrenal cortex. However, in 1948, Sayers and colleagues described a method of assay based on the marked depletion of ascorbic acid from the adrenal cortex following corticotrophin administration. This method was found to be precise, convenient and economical, and at present is the official method of corticotrophin assay. (Adopted by the Committee on Biological Standardisation of the World Health Organisation). It is performed on hypophysectomised rats, and the potency of a preparation is compared with that of an international standard, which is arbitrarily assigned the potency of 1 unit per mg.

A large number of other methods of assaying corticotrophin have

been proposed, but they have not been generally accepted. It has been argued that corticotrophin should be measured by its principal effect, that is, steroid production, and assays based on this property have been devised, both *in vivo* (Guillemin *et al.*, 1950) and *in vitro* (Saffran and Dayikes, 1953) but the convenience and precision of these methods have not equalled those of Sayers Assay which remains the standard method. The parallelism of ascorbic acid depletion to steroidogenesis has been questioned at times (Guillemin *et al.*, 1950; Li, 1953) but more recent work confirms that potency arrived at by Sayers Assay gives a true indication of the steroid hormone producing potency (Hughes and Roberts, 1960).

Thus the term "corticotrophin" is now generally taken to mean "a substance active in Sayers Ascorbic Acid Depletion Test", and this property is considered to be equivalent to its steroidogenic activity. But in a more general way any substance originating in the anterior pituitary gland which influences the adrenal cortex in some way is eligible for the description "corticotrophin" (Guest-Dunne and Young, 1954).

B. Preparation of corticotrophin.

In 1933 Collip and co-workers showed that the pituitary substance which acts on the adrenal cortex is of protein nature. In the subsequent ten years a large amount of work was done in an effort to purify this substance, and the various

extraction procedures used depended on the methods of classical protein chemistry (e.g., Fevold et al., 1940; Choy et al., 1940; Bates et al., 1940). A major advance in this field was made when Lyons (1937) introduced acetone and strong hydrochloric acid for the extraction of pituitary glands. This initial extraction procedure is efficient and has been used by Li et al., (1943) and Sayers et al., (1943), who simultaneously but independently obtained homogeneous proteins from sheep and pig pituitaries, respectively, which they claimed to be the true hormone. Both groups found the molecular weight of their preparations to be approximately 20000, and it is often referred to in literature as the "protein hormone".

A further advance came when Payne, Raben and Astwood (1950) described a more efficient method of preparation of corticotrophin, and their procedure is outlined in Fig. 2. As in previous methods a pituitary powder was first extracted with acidic solvents and stepwise salting out resulted in a corticotrophin concentrate. Such preparations were supplied commercially until the early nineteen fifties and are referred to here as "crude ACTH". The advance introduced by Payne and co-workers was the finding that a ten-fold concentration of corticotrophin can be achieved by adsorption of the crude preparation on cellulose. It was later found that oxycellulose (Astwood et al., 1951) or other cellulose derivatives were more efficient for this purpose than cellulose itself. The corticotrophin preparation

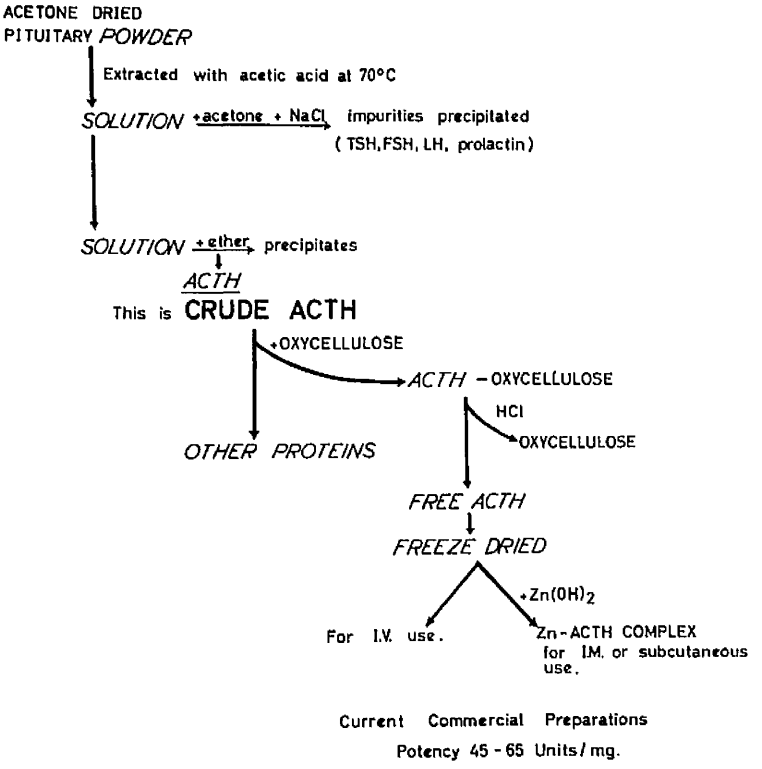


Fig. 5. Outline of the procedure used to prepare corticotrophin. Crude ACTH is an intermediate product in the preparation of the current commercial preparations.

obtained in this way was many times more active than the protein hormone of Sayers or Li, the potency being approximately 30 I.U. per mg. Preparations such as these are now exclusively supplied for general use, as shown in Fig. 5.

Although at present the only source of corticotrophin is the mammalian pituitary gland, partial synthesis has been achieved by the organic chemists. Hofmann and colleagues (1961) have synthesised a polypeptide containing the first 23 amino acids out of the total of 39 contained by corticotrophin. They found that this peptide had full activity when assayed by the ascorbic acid depletion test. It was also found to have an effect on body fats similar to that of corticotrophin (Boright et al., 1962).

C. Nature of corticotrophin and fractionation of corticotrophin activity.

The finding of Payne, Raben and Astwood (1950) that a preparation of corticotrophin can be obtained which has more than 100 times the potency of the preparations obtained by Li and Sayers in 1943, made it unlikely that the protein hormone has any true biological significance. This conclusion was further strengthened when polypeptides were obtained by acid or papain hydrolysis of pituitary extracts which had some 100 times the potency of the earlier protein products but molecular weight was only 1000-2500 (Morris and Morris, 1950; Loeh et al., 1950; Brink et al., 1952).

During the last decade evidence has accumulated that corticotrophin can be separated into various fractions which differ from one another in some physico-chemical detail which allows their separation. Thus pituitary extracts have been subjected to several types of treatment in order to prepare more potent preparations.

(a) Solvent partition and countercurrent distribution. Hess and his colleagues (1952) were the first to apply countercurrent distribution to corticotrophin and they showed that corticotrophin activity can be concentrated in this way. Bell and co-workers in American Cyanide Company (Bell et al., 1956; Shepherd et al., 1956) used these methods to obtain seven highly purified peptides, all active in the Sayers Assay, which they distinguished by prefixing the word corticotrophin with the letters of the Greek alphabet. The principal component is β corticotrophin, and the American Cyanide Company workers were able to work out the amino acid sequence of this peptide (Shepherd et al., 1956). It consists of an unbranched chain of 39 amino acids but fragments containing the first 24 amino acids are also fully active.

(b) Acid and peptic hydrolysis. In the original publication of Li et al., (1943) it was stated that the biological activity of the protein hormone was retained after treatment with pepsin

with pepsin or acid. Such treatment has been employed by various groups of investigators to prepare highly purified peptides with corticotrophin activity (e.g., Brink et al.,1952). The pepsin treated preparation is called corticotrophin B, while the untreated substance can be distinguished as corticotrophin A. These hydrolysed preparations can then be further purified using other methods mentioned here, such as countercurrent distribution or ion exchange resins. White and Pierce (1953) used such methods to obtain different active corticotrophin factors (Corticotrophin I and II). While such preparations were of high potency and active in clinical trials, the multiplicity of potent factors isolated after hydrolysis give no clue as to the situation under natural conditions.

(c) Column chromatography. Dixon et al., (1951) were the first to apply ion exchange methods to purification of unhydrolysed corticotrophin and obtained two main and two subsidiary fractions by this means. These authors suggested that there exists more than one corticotrophin A, i.e., before hydrolysis or other treatment of the native substance. They used Amberlite IRC-50 (a polymer of methacrylic acid cross linked with divinyl benzene) for chromatography in sodium phosphate buffer and a typical pattern of resolution of corticotrophin (Dixon and Stack-Dunne, 1953) is reproduced in Fig. 6. The main component active in mayors test has been designated corticotrophin A_1 other components which are less retarded are A_2 and A_3. Fraction A_4

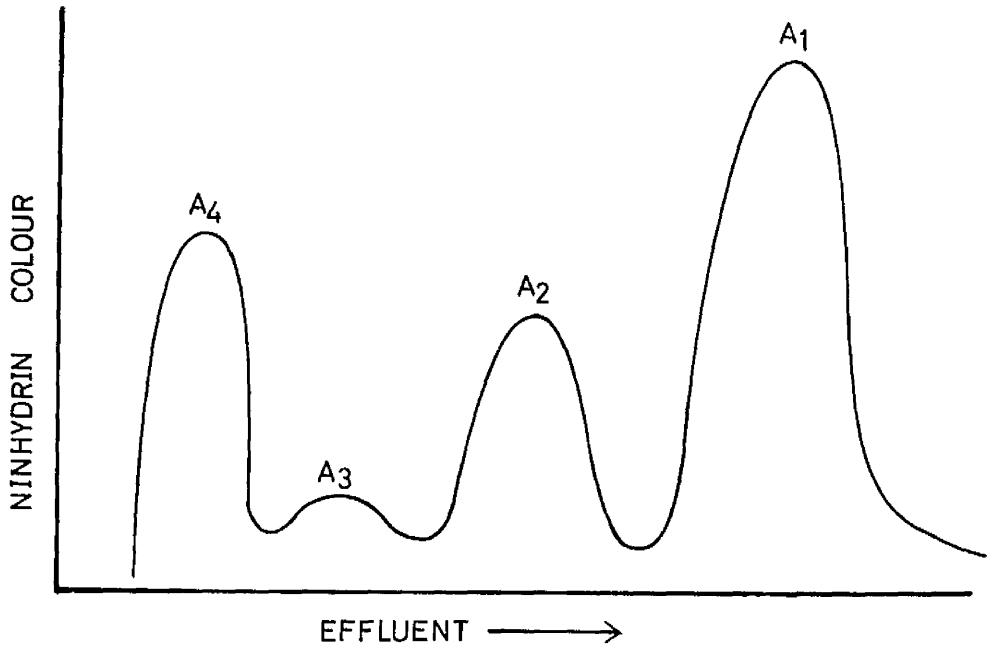


Fig. 6. Pattern of elution of oxycellulose purified corticotrophin from Amberlite IRC-50 column. (Dixon and Stack-Dunne, 1955).

is the unretarded protein material and further protein material becomes firmly attached to the resin of the column and does not appear in the eluate. Dixon and co-workers (1951 b) have also observed that while principal ascorbic acid activity was associated with the slow moving A_1 and A_2 fractions, the activity as shown by "adrenal repair" test travelled fast and was present in the A_3 and A_4 fractions to a large extent. It was proposed by this group of workers that there are separate pituitary factors with different effects on the adrenal. One was designated the "ascorbic acid factor", the other "adrenal weight factor" (Young, 1951).

Column chromatography was also used to prepare pure corticotrophin from human pituitary glands (Lee et al., 1959). The oxycellulose purified extracts were chromatographed successively on columns of a cellulose derivative, and this resulted in a homogeneous 39 amino acid peptide with a potency of 26 units per mg. The differences from the corticotrophins of ox, sheep and pig are very small, minor re-arrangements occur only in the 25-32 portion of the molecule.

It was noted above that the 23 amino acid peptide synthesised by Hofmann et al., (1961) has full ascorbic acid potency. On the other hand, the peptide synthesised by Li et al., (1960) which contains the first 19 amino acids of corticotrophin has only 30% of the biological activity (both by Sayers and Safiran Assay) of native cortico-

trophin. It has also been observed (Li et al., 1955; Shepherd et al., 1956) that in all species investigated, the first 24 amino acids are in identical sequence. This shows that only 20-23 amino acids of the 39 amino acids of corticotrophin molecule are essential for biological activity, and explains why many different fragments have biological activity. The possibility remains, however, that some of these different fragments may also be present in the adenohypophysis, and further, that their biological effects may differ qualitatively or quantitatively.

D. Control of corticotrophin secretion.

The trophic hormones of the pituitary are secreted ordinarily not at any constant rate, but in amounts varying with the physiological state of the animal. Thus corticotrophin secretion is thought to be considerable only under certain specific conditions. It had been observed very early (Stilling 1898) that removal of one adrenal results in considerable hypertrophy of the remaining gland. This, so called "compensatory hypertrophy", was later shown to be due to the intervention of the adenohypophysis since it does not occur in hypophysectomized animals (Ingle and Higgins, 1938) It can be explained by the hypothesis that lowered blood levels of adrenal steroid hormones stimulate the anterior pituitary to secrete increased amounts of corticotrophin, and is often compared to "servo" or "feedback" mechanisms.

The second set of conditions under which the release of corticotrophin is increased can be grouped together as "stress" as defined on page 4. It was at first thought that stress causes greater utilisation of the adrenocortical hormones by tissue cells, and the "servo" mechanism caused corticotrophin secretion (Sayers and Sayers, 1948). Another theory (Long, 1950; 1952) was that increased secretion of adrenaline in response to stress causes release of corticotrophin from the pituitary. But it is now known that release of corticotrophin from the pituitary is chiefly caused by a polypeptide hormone, sometimes called CRF (the corticotrophin releasing factor), which is elaborated in the median eminence of the hypothalamus and carried to the adenohypophysis by the hypophyseal portal blood vessels (Harris, 1955; Saffran and Saffran, 1959).

4. Action of corticotrophin

A. Endocrine actions.

The effects of corticotrophin on the adrenal may be observed either after administration of exogenous corticotrophin or after application of stress to an intact animal, in which case endogenous corticotrophin is released.

(a) Rapid increase in steroid hormone output. This is generally regarded as the principal physiological action of corticotrophin and occurs within a few minutes after its administration. For instance, Ingram and Nelson (1950) detected an increase in corticosterone output in adrenal venous blood four minutes after an intravenous injection of corticotrophin. The increase is also observed on perfusion of intact adrenal glands (Vogt, 1947; Hechter, 1949), and when adrenal tissue containing intact cells is incubated in a suitable medium (Saffran et al., 1952). The corticotrophin effect is not seen when the adrenocortical cell is disrupted, as by homogenising or alternatively freezing and thawing of the gland (Schiebeler, 1954; Haynes et al., 1954).

The increase in steroid output due to corticotrophin is very marked but rather variable, and on the average a five-fold increase over the basal level is seen, though it tends to be more marked in perfusion experiments than in adrenal slices.

(b) Corticotrophin influences adrenal weight. This was the first

demonstrable effect of pituitary preparations on the adrenal (Smith, 1930) and for twenty years was the basis of most tests of corticotrophin activity (Adrenal Weight Maintenance, Adrenal Repair). In normal rats, increase of gland weight of 300% has been obtained (Davidson, 1937). This increase in weight is known to be due both to hypertrophy of all the cells of the cortex (Houssay et al., 1933; Emery and Atwell, 1933) and to hyperplasia (Moon, 1937).

(c) Metabolic changes in the adrenal cortex. Addition of corticotrophin results in marked increase in oxygen utilisation by the adrenal cortex. This effect is seen both in vivo (Carpenter et al., 1946) and in vitro (Saffran et al., 1952; Haynes et al., 1953). Corticotrophin also increases the rate of incorporation of radioactive phosphorus into organic compounds of the adrenal (Gemzell, 1948).

(d) Ascorbic acid depletion. Since the isolation of ascorbic acid from the adrenal cortex by Szent-Gyorgyi (1928), numerous investigations have been made in an effort to determine the significance of its occurence in a high concentration in this organ. This has as yet not been achieved, but in 1944 Sayers and co-workers reported that administration of corticotrophin results in a marked decrease in the adrenal ascorbic acid concentration. Within one hour, levels of approximately 50% of the former concentration are seen and thereafter the ascorbic acid level slowly returns to normal. The adrenal

depletion of ascorbic acid is due to the release into the blood stream (Vogt, 1948) and it appears in the adrenal venous blood of the rat within one minute of corticotrophin administration, that is, before corticosterone increase can be detected (Lipscomb and Nelson, 1960).

(c) Decrease in cholesterol stores. Cholesterol is lowered to 50% of its concentration in the rodent adrenal following administration of corticotrophin or application of stress to the animal (Sayers et al., 1944). This finding was confusing to the early workers who had established (Smith, 1930) that hypophysectomy results in the loss of lipid and degeneration of the zones reticularis and fasciculata. This can be explained, however, on the supposition that while large amounts of corticotrophin cause a decrease in adrenal cholesterol, small steady amounts might be necessary for the survival of the two inner zones of the adrenal cortex.

(d) Histological effects. The effects of corticotrophin on the adrenal cholesterol are paralled by changes in the histologically observed sudanophilic substances, probably because these are principally cholesterol compounds. The lipid depleted cells of the zona fasciculata acquire a distinct appearance after haematoxylin and eosin staining. These and other changes have been studied in great detail in the human adrenal cortex by Symington (Symington et al., 1950; Symington, 1962). In particular he observed that after exogenous

corticotrophin administration the greatest changes are seen in the zona fasciculata. The normal lipid laden "clear" cells now appear compact and full of eosinophilic cytoplasm. Mitochondria are increased in number, ribonucleic acid is obtainable, and a number of enzymes can be demonstrated histochemically, for instance, alkaline phosphatase and succinic dehydrogenase. The appearance of the cells of the zona fasciculata is now very similar to the cells of the normal zona reticularis.

Histological effects of corticotrophin administration have also been closely studied in hypophysectomised animals (e.g., Sayers, et al 1951). When the adrenal is allowed to atrophy following hypophysectomy and corticotrophin is then administered, the following effects are seen:-

(i) The width of the cortex increases.

(ii) The remaining lipid becomes distributed in fine droplets and more uniformly throughout the zona fasciculata.

(iii) The subglomerular endocrinophobic zone becomes less prominent.

(iv) Zona reticularis becomes reconstituted.

These cytological changes, may begin as early as 10 hours after a single injection of corticotrophin and persist for several days (Miller and Nadle, 1942).

(c) Effects of corticotrophin on adrenal blood flow. The picture of adrenal vasculature presented by Flint (1909) was that of a static

and rigid vascular arrangement. But it has now been shown for several species that administration of corticotrophin results in an increased blood flow through the adrenal gland. For instance, such an effect was demonstrated for steer (Balfour, 1959) rat (Holtzbauer and Vogt, 1957) and sun adrenal (Symington et al., 1958). This effect may be compared to the situation in skeletal muscle when the number of patent capillaries varies with the state of activity, being greater when the muscle is at work (Krogh, 1929). This has been shown to apply to the mouse adrenal by Coreh and Grollman (1941) who in a descriptive histological study found that hyperactivity of the adrenal gland results in:

(i) an increase in the number of capillaries of the cortex,

(ii) an increase in the diameter of at least a part of the capillary bed.

Harrison and Daey (1960) confirm such findings using microradiography but they are of the opinion that the alterations in cortical vascularisation are secondary to the effects of corticotrophin on the adrenal cells. This is, however, not established as they give no evidence for this opinion.

D. Extra adrenal actions of corticotrophin.

It was thought until recently that corticotrophin, although brought by the blood stream to all the tissues of the body, exerted its action exclusively on the

adrenal cortex. However, evidence has been presented that corticotrophin is capable of influencing metabolic processes in tissues other than the adrenal cortex, both *in vivo* and *in vitro* (Engel, 1957; White and Engel, 1958; Hollenberg *et al.*, 1961). The validity of this evidence has depended on the demonstration that these actions of corticotrophin occur in the absence of adrenal cortex and that they are not due to contamination with the other known pituitary factors. Perhaps the most convincing support for the capacity of corticotrophin to act on a tissue other than the adrenal cortex is the demonstration that corticotrophin promotes the release of fatty acids from adipose tissue *in vitro* (White and Engel, 1958).

It is interesting to note that this extra adrenal lipolytic action of corticotrophin may be very similar to its adrenal lipid depleting action and it is possible that the mechanism of action is identical in each case.

C. Possible mechanisms of corticotrophin action.

Little is known about the mechanisms underlying the various effects of corticotrophin listed above, and the principal speculations concern the mechanism of the increase in hormone production. Saba (1960) made an excellent review of such theories and he discusses five possibilities.

(1) Corticotrophin promotes steroid release, increased steroidogenesis being a consequence of the lowered intracellular hormone concentration. The observation of Haynes and Berthet (1957) that

intracellular concentration of free steroids increases after corticotrophin makes this theory untenable.

(2) Synthesis of an enzyme protein concerned in steroidgenesis is increased. Such increases have been noted (Grant, Symington and Duguld, 1957), but they occur later in time than steroidogenesis. New protein synthesis does not occur *in vitro* (Koritz *et al.*, 1957) in experiments on rat adrenals lasting a few hours, though steroid production is taking place. Another theoretical objection is the extreme rapidity with which steroids appear after corticotrophin administration (1-4 min.).

(3) Stimulation of the enzyme reactions in the biosynthetic sequence (see Fig. 3). This was demonstrated by Stone and Hechter (1954) who used perfused bovine adrenals and showed that corticotrophin stimulates greatly the conversion of cholesterol to progesterone, but not the other steps in the sequence. Currently available evidence indicates that the rate limiting step is the conversion of cholesterol to Δ^5-pregnenolone rather than the subsequent oxidation of pregnenolone to progesterone.

It does not necessarily follow that the stimulation of this reaction is due to the increased activity of the enzyme catalysing this step. Increased availability of a cofactor or the transport of the substrate to a correct place within the cell would have the same effect.

(6) Regulation of the rate of formation of a cofactor. Haynes and Berthet (1957) were able to show that corticotrophin rapidly increases adrenal phosphorylase activity and they postulated that a reaction sequence initiated in this way results in the increased production of $NADPH_2$ which is necessary for corticosteroid production (Fig. 7). Haynes (1958) has further found that corticotrophin causes increased accumulation of adenosine-3',5'-monophosphate (3',5'-AMP) in adrenal slices and that this compound stimulates adrenal phosphorylase activity. The integrated scheme proposed by Haynes for the mechanism of corticotrophin action is shown in Fig. 7. It may be significant that the rate limiting step in corticosteroid production (i.e., cholesterol ⟶ pregnenolone) is now known to have an absolute requirement for $NADPH_2$ (Halberstam et al., 1961).

The importance of $NADPH_2$ for corticosteroid production has also been shown by Koritz and Peron (1958) who used *in vitro* systems. They found that the addition of $NADPH_2$ or systems which produce it (e.g., G-6-P and NAMP) to quartered rat adrenals results in a marked stimulation of the corticosteroid production. However, they found that addition of corticotrophin and a $NADPH_2$ producing system together lead to corticosteroid production greater than with either of the additions alone. This suggests that corticotrophin has an action on the adrenal which is additional to its ability to increase $NADPH_2$ production. Koritz and Peron postulate that this other action may be

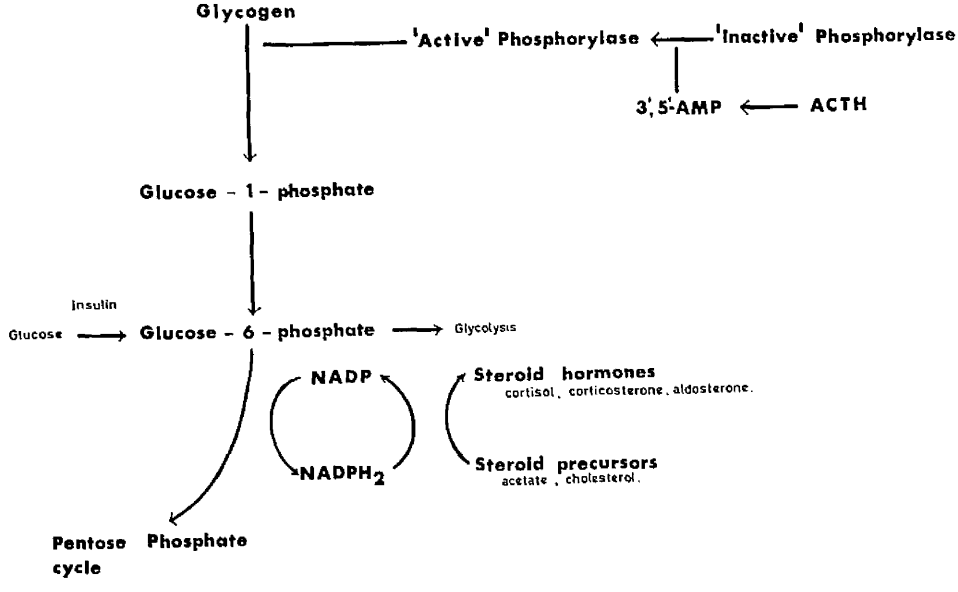


Fig. 7. Scheme outlining the mechanism of corticotrophin action suggested by Haynes (1958).

to make a precursor available for biosynthetic reactions.

(5) "Integration". This term is employed by Saba (1960) to mean that corticotrophin can influence reactions by making available the substrate at the right place within the cell. For instance, the reactions cholesterol ⟶ pregnenolone and 11 β hydroxylation take place within the mitochondria, while the other biosynthetic steps take place within the microsomes or the cell sap. It would seem that there must be a movement of substances to and from the mitochondria, but Hayano et al., (1956) postulated a "mitochondrial complex", all reactions taking place on the surface of the mitochondria activated by enzymes both from within and from the outside. It is suggested that corticotrophin can influence the integration of such a complex. The importance of cellular organisation for corticotrophin action is also shown by finding that corticotrophin has no action when the cell is disrupted (e.g., Reich and Lehninger, 1955).

A further theory regarding the mode of corticotrophin action, not discussed by Saba (1960), is the suggestion that the primary event is an increase in the adrenal blood flow, with all other effects being consequences of this action (Hechter et al., (1951). This does not explain corticoid production in vitro.

Fiala and Glinsmann (1961) have shown that one hour after corticotrophin stimulation of the adrenal cortex there was an increase in simple ribonucleotides which contain high energy phosphate bonds

(e.g., adenosine triphosphate). They postulate that "the actions of corticotrophin are mediated through a central factor of changed level and distribution of acid soluble ribonucleotides". Since such compounds are involved in the synthetic functions of the cell and can be precursors for ribonucleic acid synthesis, this theory seems particularly applicable to the mechanism of the adrenal growth seen after corticotrophin administration.

D. Time factors in corticotrophin action.

A distinction can be made between two apparently different effects of corticotrophin on the adrenal cortex. There is a rapid effect which is evident within a few minutes, and a slow action which becomes apparent only after several days or weeks of treatment.

The rapid effects include ascorbic acid and corticosteroid output by the adrenal, and the increased blood flow. On the other hand cholesterol depletion of the adrenal cortex takes 3 hours to develop (Sayers et al., 1946), and cytological changes are usually seen after at least 12 hours. To distinguish these effects the terms "acute" and "chronic" are sometimes used, but it is hardly within the normal usage of the word "chronic" to refer to effects seen after only one or two days of corticotrophin administration.

A curious example of the effect of the time factor on adrenal performance is furnished by the study of the ratio of cortisol to

corticosterone in adrenal effluent blood. For instance, administration of corticotrophin results in an immediate increase in the output of these hormones, but in the same proportion as found before corticotrophin administration. Several days of treatment with corticotrophin, however, results in an increasing preponderance of cortisol over corticosterone (Grant, Forrest and Symington, 1957).

We can postulate, therefore, that corticotrophin has two different actions which become apparent at different times. But a more likely hypothesis is that in addition to the phenomena connected with the sudden output of steroids, other cellular mechanisms are set in motion which do not become evident till later, and which prepare the adrenal cortex to deal more efficiently with future demands on its secretory capacity. It is interesting to note that electrical activity is of such a biphasic pattern in the rat adrenal gland following corticotrophin administration. A sudden sharp peak on the electrogram is followed by a more slowly developing late reaction (Krunkemper and Reichertz, 1959).

5. SPECIAL PROBLEMS OF THE HUMAN ADRENAL CORTEX

Man is a poor experimental animal. The conditions of the experiment can seldom be fully standardised and the aim of the experiment often becomes secondary to considerations of the patient's welfare. For this reason studies on human tissues are greatly outnumbered by experiments on laboratory animals, and our knowledge of the rat adrenal, for instance, far exceeds our understanding of the human adrenal. But the human adrenal cortex is quite characteristic of man, and results obtained with other species cannot be applied without direct confirmation on the human tissue. This understanding of the normal function and structure of the adrenal cortex is essential to any attempt to understand adrenal derangements as manifested by the various adrenal syndromes.

The principal difficulty in the study of the human adrenal is the unavailability of normal glands. The post-mortem adrenals can never be regarded as normal for two principal reasons:-

(i) Post-mortem autolysis sets in extremely quickly and alters histological appearances and enzyme content of the cells.

(ii) The processes associated with death act as stress and have definite effects on the adrenal cortex.

However, glands removed very quickly after death from patients suffering sudden death can approximate in appearance to "normal" glands.

This problem has not been fully appreciated in the past, and

to the errors introduced by transferring the results of animal studies to the human tissues can be added those which were due to regarding post-mortem appearances of adrenal gland as representing the normal. The introduction of surgical removal of adrenal glands as a method of therapy for advanced breast carcinoma (see Currie, 1937) has resulted in a limited availability of adrenal glands which can be regarded as normal in many cases. Those have been used for the study of structure and function of normal human adrenal glands, principally by Symington and his group. (Symington et al., 1950; Grant, Symington and Duguid, 1937). Symington and co-workers (1955) also made a thorough study of the human adrenal gland as soon as death. The most significant findings were that while the cortex of a child dying after a severe stress shows complete lipid depletion, in adults the depletion of lipid usually occurs in a focal manner, the zona fasciculata appearing to consist of alternating areas of clear and compact cells.

The human adrenal gland is difficult to obtain in a suitably fresh state and the studies on it are often unsatisfactory for many reasons. It is felt, however, that the importance of the ultimate aim of comprehending the basis of human adrenal diseases justifies the attempts to understand the adrenal structure and function which are reported on the following pages.

S E C T I O N I

EFFECTS OF DIFFERENT PREPARATIONS OF CORTICOTROPHIN ON ADRENAL GROWTH AND STRUCTURE

In this section evidence is presented to show that some of the effects of corticotrophin are separable from one another, and indicates that extracts of the anterior pituitary gland of animals contain at least two distinct substances with an action on the adrenal cortex of man. The evidence is based on the effects of corticotrophin preparations of different degrees of purity on the adrenal weight, the mitotic activity and the histological appearances of the adrenal cortex of man. The previous work on this subject has led to inconclusive results (Stack-Dunne and Young, 1954) and all previously reported experiments were performed on laboratory animals.

In the course of the investigations designed to study the varying effects of different corticotrophin preparations it was necessary to know the normal weight of the human adrenal gland and whether the left gland is larger or smaller than the right gland. This was important because the adrenal gland removed after corticotrophin administration was always the left adrenal, and it is known that in some species there is a weight difference between the right and the left gland (see Chester Jones, 1957). Perusal of the literature showed that our knowledge on these points is in an unsatisfactory state. Table I lists a number of investigations which were primarily concerned with the question of adrenal weight in human beings, and it can be seen that there is a considerable variation in the figures

quoted for the "normal" adrenal and very little documented evidence was found as to which adrenal gland is usually the heavier one. It should be noted that all the investigations shown in Table I refer to adrenal glands obtained post-mortem. No reference to the normal weight of glands removed by operation from living patients has been found in the literature, probably because it is only recently that adrenal glands which show no abnormal features began to be removed for therapeutic reasons. Observations on the mean weight of the normal adrenal gland of females are therefore presented here, together with similar data on the adrenals obtained post-mortem.

TABLE I

The figures quoted for normal weight of human adrenal glands

Author	Average single adrenal weight quoted in g.	Comments
Arren (1894)	7.2-14.2	50 adults, over 20 yrs.
Simmonds (1898)	7	Variation from 3 to 11 g.
Delamare (1903)	5.1	Males
Delamare (1903)	4.4	Females
Simmonds (1903)	5.6	200 cases
Vierordt (1906)	5-10 g.	Collected data from earlier reports in the literature
Merkel (1915)	7.4	Adults only
Schilf (1922)	5.6	Women 30-70 years
Materna (1923)	5	Sudden death
Lucien and George (1927)	6.0	Adults
Rössle and Roulet (1932)	6.8	Males
Rössle and Roulet (1932)	6.1	Females
Quinan and Berger (1933)	4.15	Sudden death, mostly men, 50 cases
Zuckerman in "Textbook of Anatomy" Ed. Hamilton (1950)	5-10	
Gunningham's Textbook of Anatomy Ed. Brash, J.C. (1951)	7-12	Mentions "as found at post-mortem"
Gray's Textbook of Anatomy. Eds. Johnstone, T.B. & Whillis, J.	3-4	Probably based on Quinan and Berger's values

The values quoted above were based on the weight of adrenals obtained post-mortem.

II MATERIALS AND METHODS

1. Human Adrenal Cortex.

(a) Post-mortem adrenals. Post-mortem adrenals were obtained from the consecutive autopsies on 30 adult males and 30 adult females, performed on patients dying in the Glasgow Royal Infirmary. No selection of the cause of death was made and all cases were included where the post-mortem was performed within 24 hours of death. The most common causes of death were cardiovascular disease and malignant neoplasms, and death was usually, but not always, preceded by a considerable period of illness. The adrenals were carefully dissected free of adherent fat, blotted on a filter paper and weighed to the nearest 0.01 g., the right gland being kept distinct from the left gland.

(b) Operation adrenals. These glands were obtained from adult female patients undergoing bilateral adrenalectomy for advanced breast carcinoma with metastases. The patients were usually ovariectomised at the first stage of the adrenalectomy, but in some cases ovariotomy was performed some time previously. The first gland, always from the right side in the series quoted in this section, was removed without any hormone pretreatment. The left adrenal was removed after an interval of 7-10 days, depending on the clinical condition of the patient. This time the operation was preceded by the administration of corticotrophin for 4 days immediately before the operation. The

total dose of corticotrophin was 360 International Units (I.U.), 40 I.U. of a long acting preparation being given twice daily, at 6 a.m. and at 6 p.m., with the last dose on the morning of the operation; the gland was removed usually at 10 a.m.

The adrenals were trimmed free of fat and adherent tissues and weighed as before. In some cases they were incompletely removed or contained obvious metastatic deposits. These glands could not be included in the present series of weight determinations, though other observations were made on them. Some glands contained a medullary haemorrhage; it was found possible to evacuate the blood clot and weigh the gland in a high proportion of such cases.

Two small pieces of each adrenal gland showing a complete transverse cross-section were removed and put into fixative for histological studies. The remainder of the gland was used for studies reported in the next two sections of this thesis. The pieces for histology were fixed in 10% neutral formalin. One piece was dehydrated and embedded in the usual way and 6μ sections were cut and stained with Harris's haematoxylin and eosin. The other piece was embedded in gelatin and frozen sections were cut at 6-10μ, and stained with Sudan IV. The degree of lipid depletion was estimated by a method of grading used by Currie and Symington (1955). This grading is shown in Table II.

(c) Mitotic counts. Mitotic figures were looked for in

TABLE II

Grades of adrenal cortical depletion

% of cortex occupied by compact cells	Assessment
Normal cortex	0
1-25	+
25-50	++
50-90	+++
over 90	++++

(from Currie and Symington, 1955)

100 consecutive high power fields (4 mm. objective) in the adrenal cortex. Only cells which showed a stage of mitosis which could be identified with certainty were accepted as being in mitosis. Each mitotic figure seen with the 4 mm. objective was further inspected under a 2 mm. oil immersion objective, and absence of nuclear membrane but presence of chromatin sheins was looked for. The count was expressed as the number of mitotic figures found in 100 high power fields, and this is referred to as the "mitotic index". An area seen in 100 high power fields contains approximately 16000 parenchymal cells.

2. Animal Experiments.

White albino rats of Wistar strain and guinea pigs of local stock were used. The animals were all male; the rats were young adults, the guinea pigs were immature. The body weights were recorded at the time of sacrifice. 4 I.U. of a long acting preparation of corticotrophin were given morning and evening for 4 days, with an additional dose on the morning of the sacrifice. Thus each animal received 36 I.U. over that period. The dose could not be conveniently reduced as even with a tuberculin syringe the accuracy of dosage would be low using the preparations of corticotrophin available. Long acting preparations of corticotrophin cannot be diluted.

The animals were killed by a blow on the head, adrenals were quickly removed, blotted on a filter paper and weighed to nearest 0.1 mg.

Control animals were injected with a similar volume of isotonic saline and were handled at the same time as the experimental animals. All animals were housed in standard cages, 4 rats to a cage or 2 guinea pigs to a cage. The diet was the Rowett Research Institute (Aberdeen) formula 18 for rats, and formula 41 for guinea pigs.

3. Hormone Preparations.

(a) Corticotrophin. In the experiments headed "commercial corticotrophin" the preparations administered were those available to the wards in the ordinary way. Since the patients were in different surgical units, both the pharmaceutical firm and the batch of each course of injections tended to vary considerably, though Armour products were most often used. These were various batches of ACTH-Al gel, which is prepared from bovine or hog pituitaries and to which gelatin is added to retard absorption after intramuscular injection. In a few cases Crookes and ordinary commercial long acting preparations from Organon were also used. The potency in each individual case is not stated by the makers but presumably exceeds the minimum of 45 I.U./ mg. laid down by the therapeutic substances act (1955). For all other experimental groups the corticotrophin was supplied to the wards and was one of the several types of corticotrophin listed below. Only these special preparations were given to animals.

(1) "ACTH-crude". - supplied as cortrophin Z Organon, of the type which was commercially available in the early years of the last

decade, though now considered too impure for general use. Its potency is approximately one international unit (I.U.) per mg.

(ii) ACTH-A_1A_2 - this preparation was specially prepared by Organon Laboratories as described on p. 20 and Fig. 6, by subjecting to column chromatography corticotrophin prepared by the method of Payne, Rabon and Astwood (1950) but using oxycellulose (Astwood, et al., 1951) instead of cellulose. The column chromatography was carried out using carboxymethylcellulose and only fractions A_1 and A_2 were collected, as defined by Dixon and Stack-Dunne (1955). The chromatographic separation was checked by use of the resin Amberlite - IRC 50, as used by Dixon and Stack-Dunne.

The potency of this preparation is approximately 150 I.U./mg.

(iii) ACTH-A_3A_4 (p. 20). For this preparation the fast moving peaks were collected during the preparation of ACTH-A_1A_2 in Organon Laboratories. The potency is 12 I.U./mg.

All the Organon preparations described above were retarded for intramuscular use by zinc hydroxide. The units refer to subcutaneous Sayers assay (Sayers et al., 1948).

(b) Cortisone. In one group of patients currently available preparations of cortisone and hydrocortisone for oral and parenteral use were administered for purely therapeutic reasons at the discretion of the clinicians in charge of those patients.

RESULTS

1. Weight of the Human Adrenal Gland.

Table III shows the results of weighing 60 consecutive pairs of post-mortem adrenals. All causes of death are included, and all subjects were adults over the age of 30 years. The chief point investigated here, is whether one of the glands tends to be heavier than the other, i.e., is the right adrenal consistently heavier than the left adrenal or vice versa. It is seen in Table III that there is no such consistent difference between the right and left adrenal.

The second point evident in Table III is that the adrenal glands of men are of approximately the same weight as the adrenals of women.

The adrenal glands obtained at operation, if not pretreated with corticotrophin, were generally noticeably smaller than the adrenal glands obtained at post-mortem. This is shown in Table IV, where the mean weights of these glands are compared. In each case all the glands are from the right side of women between the ages of 30 to 70 years. Since, for technical reasons, surgeons prefer to remove the right adrenal first, only a few adrenals from the left side were obtained from cases where the operation was not preceded by contralateral adrenalectomy. For this reason a similar comparison cannot be made of adrenal glands from the left side. It is clearly seen, however, that at least as regards the right adrenal, there is a marked and significant difference between the weights of adrenal glands obtained

TABLE III

Weights of human post-mortem adrenal glands

Sex	Side	Number of cases	Mean gland weight in g.	Standard error of mean in g.
Males	R	30	5.95	± 0.28
	L	30	6.03	± 0.37
	Combined weight		12.0	
Females	R	30	6.02	± 0.31
	L	30	5.78	± 0.27
	Combined weight		11.8	

The differences between the mean values shown above are not statistically significant.

Table IV

Comparison of the weight of adrenal glands obtained at post-mortem and at operation

Glands	Number of glands	Mean weight of a single adrenal in g.	Range, g.	Standard error of mean	Significance of the difference between the means
Post-mortem glands	30	6.02	3.2-12.0	0.31	$P < 0.001$
Operation glands	30	4.01	2.7- 6.3	0.22	

In each series, the glands are from the right side of females in the same age group (30-70 years).

at post-mortem and at operation. The post-mortem glands are heavier and show much greater variation in weight. It was also noted that the heaviest adrenals seen at post-mortem were from cases of acute fulminating infections; e.g., gross acute pyelitis with pyaemia - combined adrenal weight 19.9 g., peritonitis with a large subphrenic abscess - 23.5 g. On the other hand adrenals from a powerfully built adult male who suffered accidental death weighed together only 8.0 g. However, at present the series is too small to classify the adrenal weights according to the cause of death.

2. Effects of Preparations of Corticotrophin of Varying Purity on the Human Adrenal Cortex removed at Operation.

(a) Effect of corticotrophin on the weight of the adrenal gland.

As shown in Tables V, VI and VII, ACTH-A_1A_2 causes only a slight increase in the weight of the human adrenal gland (+20%) while crude ACTH has an effect on adrenal weight which is more than 5 times as marked as that of ACTH-A_1A_2 (+110%). Various commercial preparations of corticotrophin have an inconstant effect falling between these two extremes. Experiments in which ACTH-A_3A_4 was used showed that this preparation has no more adrenal growth promoting activity than ACTH-A_1A_2. Table VIII shows that when cortisone was given instead of corticotrophin adrenal weight shows no alteration.

The difference between ACTH-A_1A_2 and ACTH-crude was also investigated on the adrenals of laboratory animals. Table IX shows the

TABLE V

Effects of some commercial preparations of corticotrophin on human adrenals

	Name of patient	Corticotrophin administered	Adrenal weight, g.			Mitotic index	Histological assessment
			1st op.	2nd op.	Increase		
1.	Keogh	ACTH-AR gel				1 → 7	0 → +++
2.	Willis	ACTH-AR gel	3.8	7.6	+100%	0 → 4	0 → +++
3.	Kennedy	ACTH-AR gel	6.3	9.2	+46%	3 → 6	0 → ++++
4.	Reid	ACTH-AR gel	6.0	8.2	+37%	0 → 15	0 → ++
5.	Cameron	Crookes' ACTH	5.0	7.4	+48%	1 → 6	+ → +++
6.	Grant	ACTH-AR gel	-	7.7	-	2 → 10	0 → +++
7.	Young	Organon Zinc Cortrophin	3.0	6.5	+117%	0 → 10	0 → +++
8.	Logan	Organon Zinc Cortrophin	3.8	5.3	+40%	1 → 8	+ → ++++
	MEAN		4.5	7.7	+71%	1 → 8.5	0 → 3 or 4+

In each column the first figure is the value for the gland obtained at the first operation which was not preceded by corticotrophin administration to the patient. Second value from the gland removed after corticotrophin was administered. 40 I.U. were given twice daily by intramuscular injection for 4 days. Last injection 4 hours before the operation.

TABLE VI

Effects of ACTH-A_1A_2 on human adrenal glands

	Patient	Adrenal weight, g.		Mitotic index	Histological assessment
		1st op. 2nd op.	Increase		
1.	Jamieson	4.5 ⟶ 5.4	+20%	0 ⟶ 4	1+ ⟶ 2+
2.	McDougall	2.8 ⟶ 3.6	+28%	0 ⟶ 1	1+ ⟶ 1+
3.	Vines	3.8 ⟶ 4.5	+18%	1 ⟶ 3	0 ⟶ 1+
4.	Bell			0 ⟶ 4	0 ⟶ 2+
5.	Lees	4.9 ⟶ 5.8	+18%	0 ⟶ 2	0 ⟶ 1+
	MEAN	4.0 ⟶ 4.8	+20%	0.2 → 2.8	0 ⟶ 1+

40 I.U. of ACTH-A_1A_2 were given twice daily by intramuscular injection for 4 days. Last injection 4 hours before the operation.

TABLE VII

Effects of crude ACTH on human adrenal gland

	Patient	Adrenal weight, g.		Mitotic index	Histological assessment
		1st op. 2nd op.	Increase		
1.	Dalling	4.6 ⟶ 8.3	+81%	0 ⟶ 7	0 ⟶ 4+
2.	Henry	3.2 ⟶ 8.5	+162%	0 ⟶ 20	1+ ⟶ 4+
3.	Henderson	5.0 ⟶ 8.9	+75%	1 ⟶ 35	0 ⟶ 4+
4.	Kerr			4 ⟶ 6	0 ⟶ 3+
5.	Anderson	4.1 ⟶ 6.9	+70%	0 ⟶ 7	1+ ⟶ 3+
6.	Cox	3.4 ⟶ 6.1	+81%	0 ⟶ 37	0 ⟶ 3+
7.	Klouman	3.6 ⟶ 10.3	+186%	2 ⟶ 34	0 ⟶ 4+
8.	Dorrans	4.1 ⟶ 7.6	+117%	0 ⟶ 9	0 ⟶ 4+
	MEAN	4.0 ⟶ 8.1	+110%	1 ⟶ 19	0 ⟶ 4+

40 I.U. crude ACTH were given intramuscularly twice daily for four days. Last injection 4 hours before the operation.

TABLE VIII

Comparison of adrenals removed at two stages of bilateral adrenalectomy when only cortisone was administered between the stages

	Patient	Adrenal weight, g.		Mitotic index	Histological assessment
		1st op. 2nd op.	Change		
1.	Sinclair	3.9 ⟶ 4.7	+21%	1 ⟶ 2	1+ ⟶ 2+
2.	Knox	4.1 ⟶ 4.0	-3%	2 ⟶ 1	0 ⟶ 1+
3.	Lightbody	4.0 ⟶ 3.4	-15%	3 ⟶ 2	1+ ⟶ 2+
4.	Kirpatrick	4.2 ⟶ 3.0	-29%	1 ⟶ 1	1+ ⟶ 1+
	MEAN	4.1 ⟶ 3.8	-7%	2 ⟶ 15	1+ →1+ or 2+

50-100 mg. cortisone were administered daily in the interval between the operations (7-10 days).

effect of these preparations on the adrenal weight in the rat. The difference in growth promoting activity of the two preparations is again evident, although it is not quite so striking as in the case of the human adrenal. In the rat both preparations cause adrenal weight increase, and the increase is twice as marked in the case of ACTH-crude as for ACTH-A_1A_2. It should be noted that the dosage used for the rat is relatively very much higher than was used in the human cases. Any impurity therefore, present in the ACTH-A_1A_2 preparation, would have a correspondingly greater effect.

Table X shows a similar comparison using the guinea pig and the results are almost exactly analogous to those seen in the rat adrenal. Again the crude preparation has twice the growth promoting potency of the pure preparation.

(b) Effect of corticotrophin on the mitotic activity in the adrenal cortex. Tables V, VI, VII and VIII show that administration of any type of corticotrophin results in an increase in the visible mitotic activity in the adrenal cortex, and that this increase parallels the increase in weight. Thus the administration of crude-ACTH, has the greatest effect on the mitotic index and ACTH-A_1A_2 has the smallest effect, with commercial preparations again having an intermediate effect. It can also be seen in these tables that the effect on the mitotic activity of all types of corticotrophin is inconstant.

TABLE IX

Comparison of the effects of pure and crude corticotrophin on the weight of the adrenal gland of the rat

Group	Substance injected	Number of animals	Mean body weight in g.	Mean total adrenal weight in mg. ± S.D.	Adrenal weight per 100 g. body weight	Increase over injected control
A.	None	4	300	32 ± 6.5	10.7	-
B.	Isotonic saline	8	295	41 ± 6.5	14.2	0
C.	ACTH-A_1A_2	8	280	56 ± 10.3	20.0	+36%
D.	Crude ACTH	8	290	66 ± 7.7	23.1	+68%

4 I.U. of a long acting preparation of each type of corticotrophin were given intramuscularly twice daily for four days and the animals were sacrificed on the fifth day. The difference between the means of the two control groups (A and B) is not significant ($P > 0.05$), but the difference between the means of group C and group D is significant ($P < 0.05$); the means of groups C and D are significantly higher than the mean of group B.

TABLE X

Comparison of the effects of pure and crude corticotrophin on the weight of the adrenal gland of the guinea pig

Group	Substance injected	Number of animals	Mean body weight in g.	Mean total adrenal weight in mg. ± S.D.	Adrenal weight per 100 g. body weight	Increase over control
A.	Isotonic saline	6	318	150.9 ± 8.6	47.4	0
B.	ACTH-A_1A_2	5	338	196.8 ± 22.2	58.2	22.7%
C.	Crude ACTH	6	331	236.9 ± 12.0	71.6	51%

4 I.U. of each type of corticotrophin were given twice daily for four days. The difference between weight of groups B and C is highly significant ($P < 0.01$), and both are significantly higher than the mean of group A. ($P < 0.05$).

When no corticotrophin was given (Table VIII) the mitotic index shows little change.

When the mitotic activity is high, as after crude-ACTH, it was very often observed that the mitotic figures tended to be localised in several small areas. That is, mitotic figures are not found distributed uniformly and randomly in the section but occur in particularly high density in a small portion of the cortex. Such a concentration is illustrated in Fig. 8; six mitotic figures are seen in one high power field, while the total count in 100 high power fields of this adrenal cortex was 39. In the rodent such a concentration of mitotic activity occurs in the zona intermedia (Blumenthal, 1940). In man, however, mitotic figures can be seen in any part of the cortex (Carr, 1959), though in the cases examined in the present series mitotic figures were found very infrequently in the zona glomerulosa.

No attempt was made here to evaluate the relative proportions of the various stages of mitosis. Such an evaluation is bound to be misleading since the stages of mitosis which are most easily seen are short, and prophase and telophase which last longer, are difficult to distinguish from the pycnotic nuclei in the ordinary histological sections. All stages of mitosis, however, were noted in the human adrenal cortex.

The great majority of mitotic figures in the human adrenal cortex

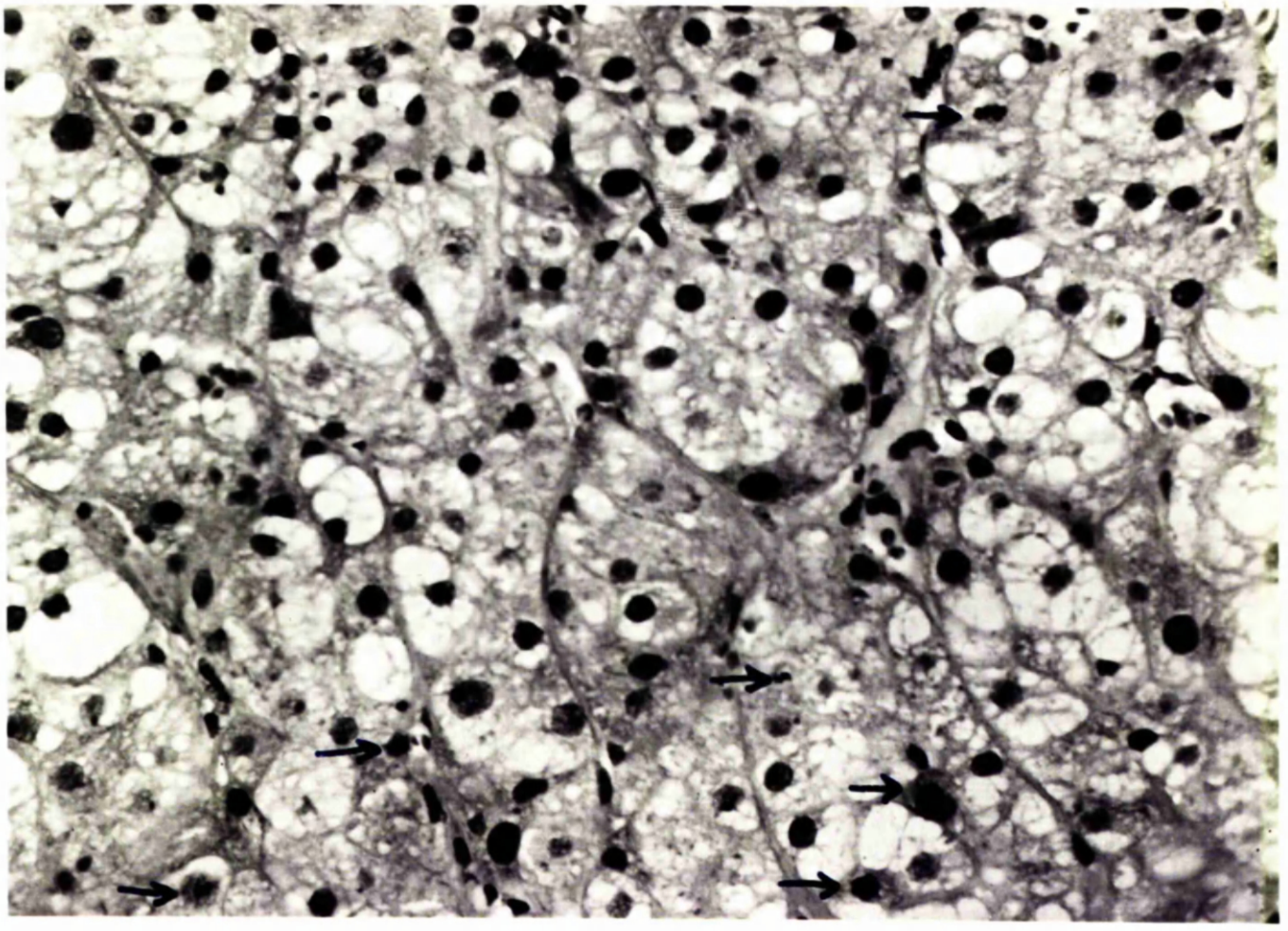


Fig. 8. High local density of mitotic figures in the human adrenal cortex. The field illustrated shows 6 mitotic figures in the adrenal cortex of a patient treated with crude ACTH. Magnification: x 375. Stained by H. and E.

were seen in the "compact" cells, as noted by Carr (1959). However, a small proportion of mitoses were clearly occuring in the "clear" cells. These clear cells, however, were in no case of the large vacuole type, but contained very small finely dispersed vacuoles.

(c) Effects of corticotrophin on the histological appearance of the human adrenal cortex. The different preparations of corticotrophin used here have varying effects on the histological appearance of the human adrenal cortex. Illustrative examples of the effect of each principal type of corticotrophin are shown in Figures 10, 11 and 12 and the appearances can be compared with the structure of the normal unstressed human adrenal cortex (Fig. 9). Commercial ACTH and crude ACTH cause marked lipid depletion, but crude ACTH causes complete (4+) lipid depletion more often than commercial ACTH. On the other hand ACTH-A_1A_2 has only slight effect on lipid depletion (Table VI). Glands examined after administration of cortisone instead of corticotrophin show slight lipid depletion. However, it should be noted that the removal of these glands was preceded by another operation (removal of the first adrenal) 7 to 10 days previously, and this is a stressing event.

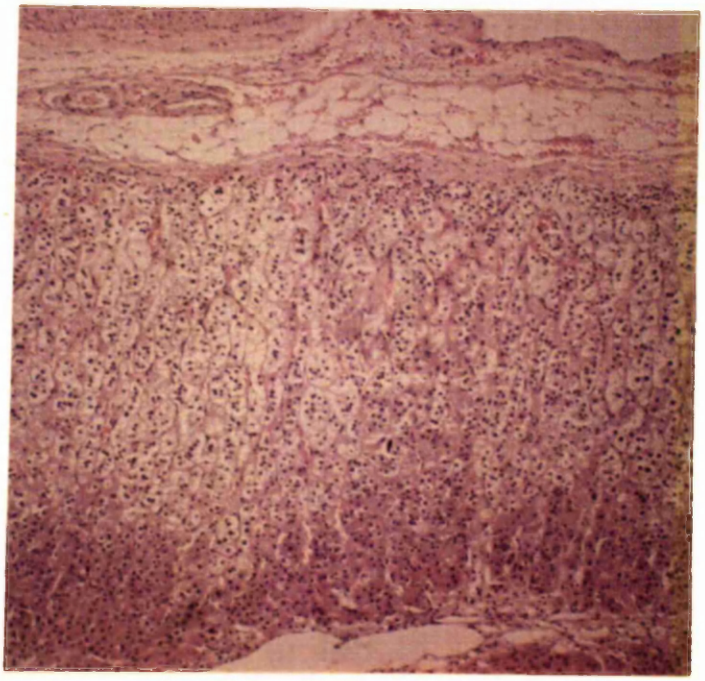


Fig. 9. Appearance of the normal unstimulated human adrenal cortex in H. and E. sections. Magnification x 50.

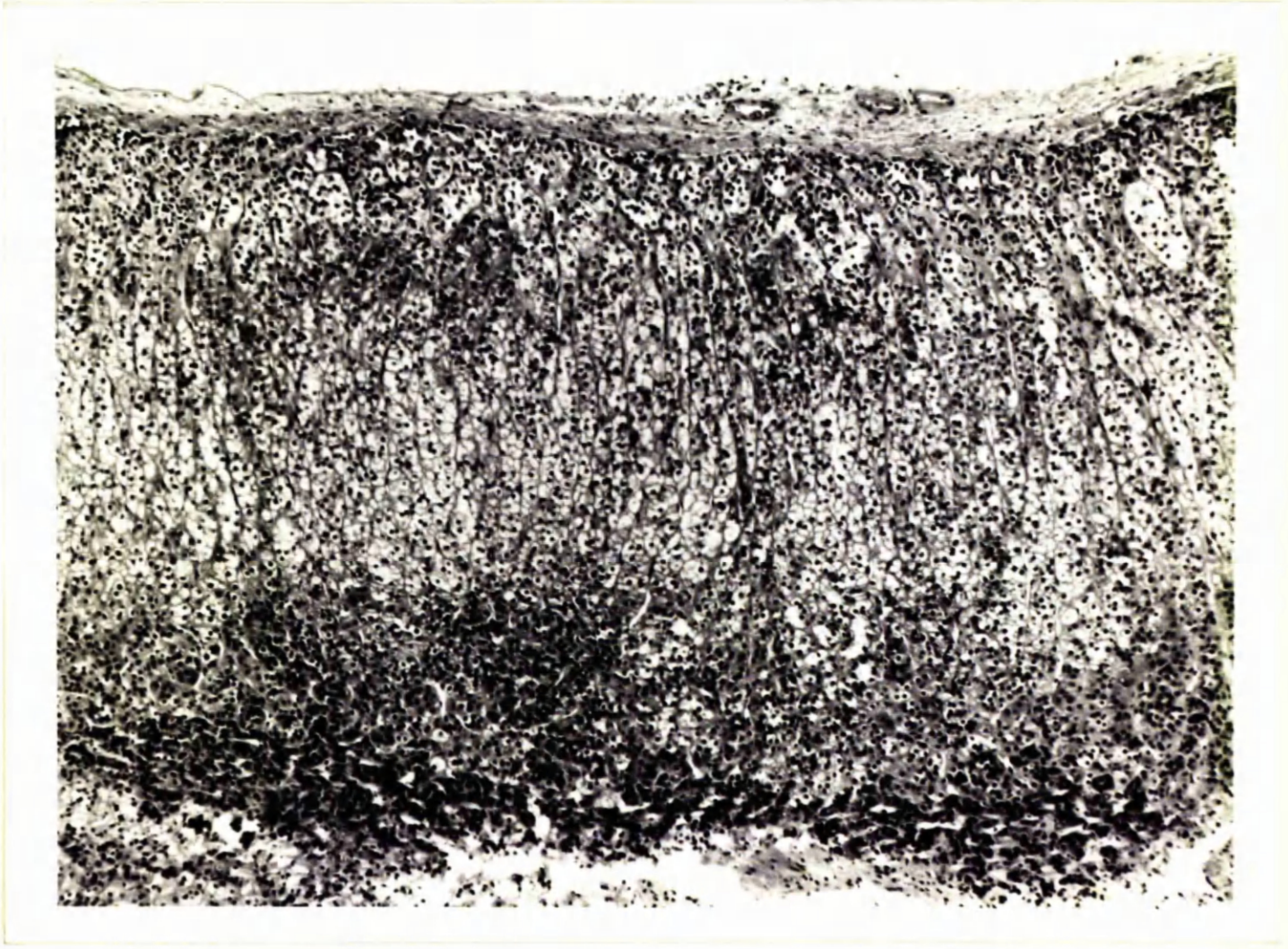


Fig. 10. Appearance of the human adrenal cortex after administration of high doses of "ACTH-A_1A_2" to the patient for 4 days. The appearances differ little from those seen in a normal gland. Magnification: x 100. Stained by H. and E.

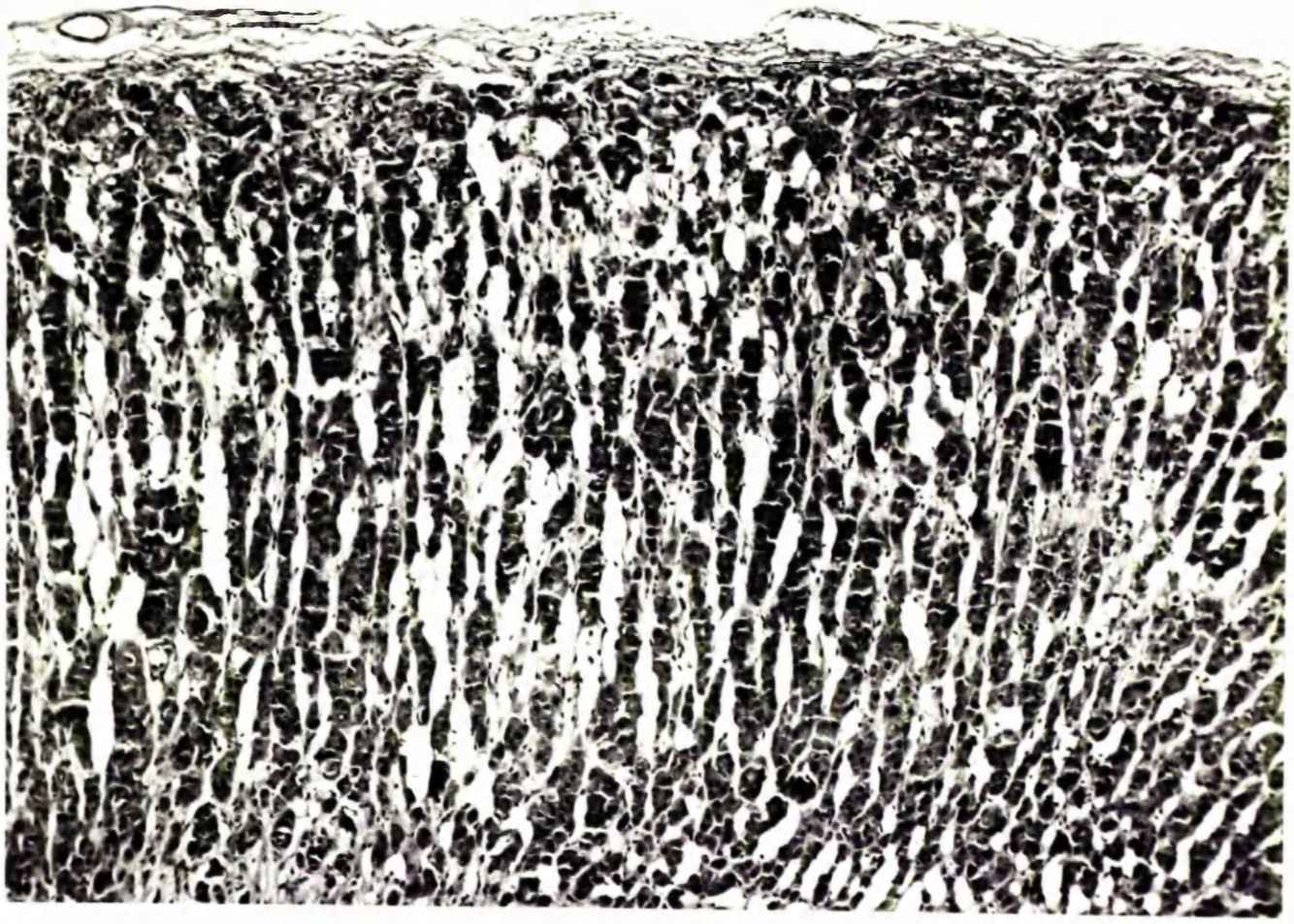


Fig. 11. Appearance of the human adrenal cortex after administration of "crude ACTH" to the patient. The compact cells now account for the thickness of the cortex. Note also dilated vascular channels. Magnification: x 90. Stained by H. & E.

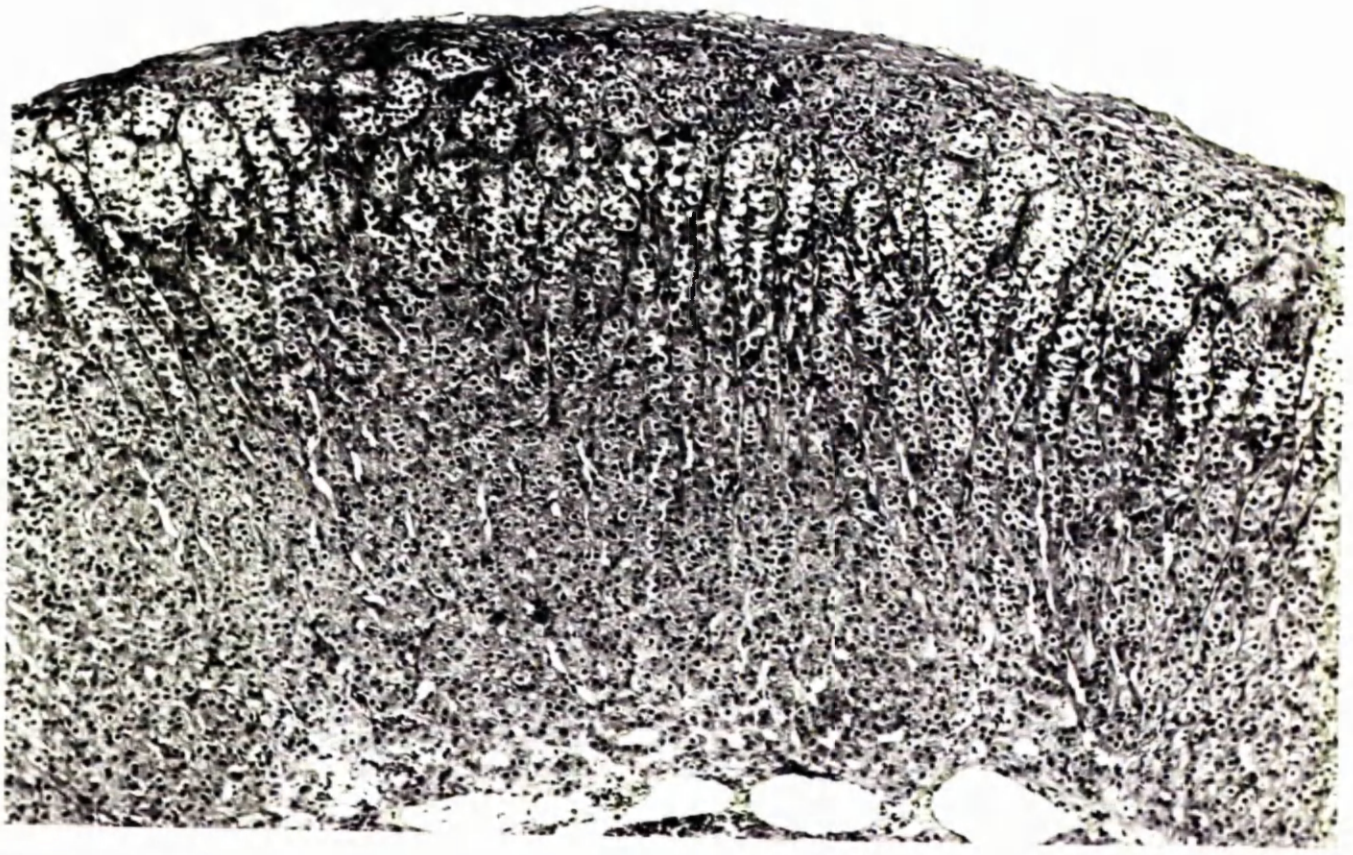


Fig. 12. Appearances of the human adrenal cortex after administration of commercial ACTH (ACTH-AR gel) to the patient. Compact cells account for approximately ¾ of the thickness of the cortex. Magnification: x 90. Stained by H. & E.

DISCUSSION

1. What is the weight of the Human Adrenal Gland?

The comparison of the weights of operation and post-mortem glands (Table III) indicates clearly that the adrenal weights obtained in a normal run of post-mortems cannot be used for obtaining the normal average weight, since the post-mortem glands are considerably heavier than the glands obtained at operation. Contrary to the previous reports (see Table I), it was found here that the mean adrenal weight of man is similar to that of woman. The figures are 6.0 g. and 5.9 g. respectively, with rather a wide range in each case (Table III).

The finding of special importance to the present studies is that the mean weight of the right adrenal does not differ from the mean weight of the left adrenal. On this point there is very little previous information in literature as regards man. Delamare (1903) states that in his series in 11 cases the right adrenal was heavier than the left, in 16 cases the reverse, and in only one case were both adrenals of the same weight. Nichols and Nicolaeva (1930) examined foetal adrenals, and in their series 61% of left glands were greater than the right gland, while only 27% of cases showed the reverse. Neither of these works quote the actual mean weights, but Quinan and Berger (1933) found that the right adrenal has exactly the same weight as the left. It is therefore reasonable to regard the change in weight of the adrenal gland observed after corticotrophin

as representing the alterations due to experimental intervention.

The mean weight of 30 right adrenals removed by operation from adult women suffering from breast carcinoma is 4.0 g. (Table IV). This is lower than the mean weight of adrenals obtained post-mortem quoted by most workers except Quinan and Berger (1933). Their mean adrenal weight of 4.15 g., for both males and females, is very close to our figure, although Quinan and Berger's adrenals were obtained post-mortem. However, their mean value is based entirely on adrenals from people suffering sudden and unexpected death. On the other hand the values obtained by Waterne and Janushkin (1927) for the mean weight of adrenals of patients dying from cardiovascular disease was 5.8 g., and from acute infections 8.4 g. Quinan and Berger concluded therefore, that processes associated with the terminal illness cause adrenal enlargement, and this conclusion is supported by the evidence presented here.

The mean figure of 4.0 g. reported here for operation glands applies only to the right gland. But since no consistent difference was found between the right and the left gland at autopsy it seems reasonable to assume that the mean weight of the left adrenal gland in women is also 4.0 g. Also, one can hazard a guess, based on the lack of significant sex difference observed in post-mortem glands, that the normal single gland weight in the male is also approximately 4.0 g.

2. Adrenal Growth.

The development and the first burst of adrenal growth up to the 4th month of intra-uterine life may be independent of anterior pituitary control (see Jost, 1962), but all subsequent increases in size are probably due to the release of pituitary factors. Thus human anencephalics who have only rudimentary pituitary tissue show a reduction in size of the adrenal gland which is evident after the 20th week of intra-uterine life. Enlargement of adrenals of adults due to stress does not occur in the absence of the pituitary gland (e.g., Bell and Samuels, 1938). Removal of the anterior pituitary of many species (reviewed by Swann, 1940) results in a prompt and marked decrease in size of the adrenal and the atrophy of the zones reticularis and fasciculata.

This indicates that the fully differentiated adrenal cell of the inner layers of the cortex (the zones fasciculata and reticularis) shows mitotic division only under the influence of the trophic factors from the pituitary. If it were known what is the mechanism of this action, perhaps many phenomena of normal and neoplastic growth would become clearer.

The increase in weight observed after the action of corticotrophin could be due to several causes e.g.,

(a) Increase in the water content of the gland, perhaps due to

(b) marked engorgement with blood.

(c) Cell hypertrophy - i.e., cells are larger, and contain more cytoplasm.

(d) Cell hyperplasia - i.e., multiplication of cells, entailing an increase in the cytoplasmic and nuclear material.

It is possible that all these causes, perhaps except (a), account for the increase in weight of the gland. "Growth" however, can best be considered to be taking place when the increase in size of an organ or tissue is taking place due to cell hyperplasia or hypertrophy (or both together). Results of Symington and Davidson (1956) and Fiala et al., (1956), showed marked increase in cytoplasmic ribonucleic acids after corticotrophin, and demonstrate that cell hypertrophy is taking place. In addition, the higher mitotic index after corticotrophin noted here and by Carr (1959) indicates that cell hyperplasia is taking place. We can, therefore, regard the two phenomena observed here, namely the increase in the adrenal weight and the higher mitotic index, as two aspects of adrenal growth.

Halberg and his colleagues (1957, 1959) have drawn attention to the fact that the frequency of mitosis in the adrenal cortex is not uniform throughout the day, but undergoes a daily rhythm. Thus in the mouse, mitoses are twice as common at midnight as they are in the early morning. This is, of course, secondary to the diurnal variation in the pituitary release of corticotrophic factors, but it emphasises that mitoses are not uniformly distributed in time.

Similar observations, that mitoses occur in definite waves in adrenal cortex and other organs have been made many years ago (Dustin, 1923). The fields illustrated in Fig. 8 show that mitoses are also unevenly distributed in space. This is also evident in the illustrations of Carr (1959) though not explicitly stated, and is analogous to the observations in rodents (Blumenthal, 1940; Deane and Greep, 1946) that mitoses are only seen in the transitional zone. This means that there are well circumscribed local conditions which are favourable for mitotic division at that time, and mitotic figures appear like mushrooms in that area. Since it appears from the discussion above that a pituitary factor is necessary for adrenal mitosis, it means that in those areas either,

(a) an additional factor or a condition is present which favours mitoses, or more likely,

(b) a suitable concentration of the pituitary factor can be selectively achieved in localised areas of the adrenal cortex. A possible mechanism for such a phenomenon would be some peculiarity of the blood supply to that area, allowing for instance, more prolonged contact of the cells with plasma constituents in that part of the cortex.

The practical point in connection with the above discussion is that mitotic counts have necessarily very low precision. If the adrenal is actively growing but removed between the waves of mitotic

activity, the mitotic index found will be erroneously low. Similarly, since statistics are based on random distribution, tests of significance are of poor validity in respect of the mitotic counts.

3. Is there more than one Corticotrophin?

Nelson and colleagues (1936) were the first to suggest the existence of more than one type of pituitary hormone with effects on the adrenal cortex. Stack-Dunne and Young (1951) and Dixon and colleagues (1951) observed a separation of the adrenal weight promoting activity from the ascorbic acid depleting activity and suggested that these suspected factors should be designated the Adrenal Weight Factor (AWF) and the Ascorbic Acid Factor (AAF). Hungerford and co-workers (1952) proposed that an eosinophil reducing component of corticotrophin is separate from the ascorbic acid reducing component, since partial inactivation had different effects on these activities. Similarly, Talbot et al., (1951) suggested that two pituitary factors control the androgen output by the adrenal cortex, and further evidence on this point has been presented by Mills et al. (1962) and Prunty (1956). However, the concept of multiple pituitary control of the adrenal has not been generally accepted. Adrenoglomerulotropin, as well as corticotrophin, is admitted to have a controlling effect on the zona glomerulosa (Farrell, 1959), but this factor is not produced by the pituitary gland but by the diencephalon region of the brain.

The results presented in this work show clearly that crude preparations of hog pituitary corticotrophin have effects on the human adrenal cortex which are not obtained with the most highly purified preparations of corticotrophin. Crude-ACTH and commercial preparations of corticotrophin have an effect on histological appearances which is not seen after ACTH-A_1A_2. The slight shift towards lipid depletion after ACTH-A_1A_2 is also seen when no ACTH was given (Table VIII) thus indicating that the change is merely the residual effect of the stresses associated with the first operation. Something is present therefore in the pituitary extracts, and presumably in the anterior pituitary gland, which has no activity on the depletion of adrenal ascorbic acid but causes marked lipid depletion. This suggests strongly that the anterior pituitary gland produces at least two factors with separate effects on the adrenal cortex.

The fact that commercial preparations, of intermediate purity, have effects intermediate between those seen after the crude and pure preparations, argues that there is a factor or factors which are progressively removed in the purification of the ascorbic acid depleting factor.

The weight and mitotic activity increased slightly even after the purest preparations of corticotrophin. This of course can represent the compensatory hypertrophy which follows unilateral

adrenalectomy in animals (Stilling, 1898). The increase of 20% in the adrenal weight is of the order of magnitude which would be expected on the basis of animal experiments (Brodish and Long, 1960). However, the possibility cannot be excluded that continuous and fairly prolonged action of ACTH-A_1A_2 results in some stimulation of growth.

The view that ascorbic acid depleting activity cannot be dissociated from some growth stimulation is in agreement with results reported for other highly purified preparations of corticotrophin. Lostroh and Woodward (1958) administered β-corticotrophin purified by countercurrent distribution and found that small doses given for 7 days can cause increase in adrenal weight in hypophysectomised mice. It can, of course, be argued that countercurrent distribution is less efficient in separating the corticotrophin factors, but their observation that addition of very small amounts of purified Growth Hormone potentiated markedly the increase of adrenal weight produced by β-corticotrophin seems analogous to our results. Growth Hormone by itself had no significant effects on the adrenal cortex.

Experiments on rats and guinea pigs shown in Tables IX and X demonstrate that the difference in effect of the various corticotrophin preparations is not peculiar to the human species.

4. Nature of the Additional Adrenocorticotrophic Factor.

The present work gives no clue as to the nature of the additional

pituitary factor with effects on the adrenal cortex. Stack-Dunne and Young (1931) first suggested that the Adrenal Weight Factor is associated with the Growth Hormone. Cater and Stack-Dunne (1935) found that the Growth Hormone stimulates mitotic division in the rat adrenal, and the work of Lostroh and Woodward quoted above (p. 57) and of several other investigators, supports the view that the Growth Hormone may be the additional factor with effects on the adrenal cortex. Liddle et al. (1954) have obtained a fraction from the hog pituitary associated with crude Growth Hormone which maintained adrenal weight of hypophysectomized rat without causing any steroidogenesis. Lanman and Nimerstein (1960) prepared an alkaline extract of beef pituitary with adrenal weight promoting activity which they believe could not be due to its corticotrophin content (as measured by Sayers Assay). They showed that gonadotrophin have an effect on adrenal weight, but that this effect is secondary to their action on the gonads since it is not seen in castrated animals. Growth hormone, on the other hand, appeared to have a direct action on the adrenal.

Jailer and collaborators (1957) have shown that the serum from pregnant mothers contains a substance with effects on the adrenal weight but with no activity in the Sayers test. Lanman and Dinerstein (1959) demonstrated that this is most likely due to the serum gonadotrophin content, which acts on the gonads to cause release

of steroid hormones, and these increase the adrenal weight. The adrenal weight increasing factor cannot act by such a mechanism in the experiments reported here, because all women were ovariectomised before or at the first operation.

There are therefore good grounds for supposing that the somatotrophic growth hormone has special effects on the adrenal cortex, though this cannot be taken as proven. The action of promoting adrenal growth can be looked on as a special example of its general property of promoting the growth of long bones and other tissues. Our results show that the human adrenal cortex can be acted upon by corticotrophin of high potency without showing the structural alterations which were seen after the action of another factor, or perhaps another factor synergistic with corticotrophin. By analogy this factor may be the Growth Hormone.

It is possible that the presence of two or more pituitary factors acting together on the adrenal cortex resembles the situation which exists in the action of oestrogens and progesterone on their target organs such as the uterus and the breast. Oestrogens cause the growth and the necessary preparatory changes for the action of progesterone. Progesterone causes further growth and changes leading to "secretory" activity. Similarly the adrenal may be controlled by separate factors, only one of which increases its secretory activity.

5. Significance of present findings in relation to Human Disease.

5. Significance of present findings in relation to Human Disease.

Adrenal enlargement is a feature of several adrenocortical syndromes. It is important therefore to have a firm baseline for normal weight of the adrenal in order to assess whether a gland removed by operation is normal. If "normal" values based on post-mortem glands are used, as is often the case, the adrenal weight may be considered nearer normality than is actually the case. For operation adrenals, values based on glands removed during life must be used for comparison.

It has long been a puzzle why in some cases of clinically and biochemically proved Cushing's Syndrome the adrenal gland is of normal or only very slightly increased weight and shows only minimal deviation from the normal histological appearance of the cortex; while in general it is a feature of Cushing's Syndrome that the adrenal gland is enlarged and the cortex shows marked lipid depletion. If there are two separate pituitary factors, only one of which has a marked effect on the adrenal growth and histology, this situation becomes quite easy to understand. We can postulate that the steroidogenic principle is secreted by the anterior pituitary in all cases of Cushing's Syndrome, and this accounts for the clinical and biochemical findings. In only a proportion of cases, however, the additional trophic factor is produced in excess. These cases, alone, show the adrenal enlargement and the marked lipid depletion thought

at one time to be characteristic of Cushing's Syndrome. The results presented above support such a hypothesis.

The possible dual nature of the pituitary control of the adrenal cortex is also of importance in relation to the efforts of the clinician to prevent iatrogenic Addison's disease. Adrenal atrophy, with consequent adrenal insufficiency has frequently been recognized following cessation of adrenal steroid therapy (Sallasa et al., 1953; Cope and Baker, 1955; Frazer et al., 1952). This adrenal insufficiency is presumably secondary to inhibition of the release of pituitary corticotrophin by the administered steroids (Ingle, 1938). Efforts are therefore often made to prevent this adrenal atrophy by simultaneous injection of exogenous corticotrophin during the course of steroid treatment. Such efforts are not unsuccessful in all cases. One factor in such a failure to maintain normal adrenal weight may be that corticotrophin injections are not given often enough; another possibility, however, is that the more highly purified preparations of corticotrophin which now become available do not contain enough of the adrenal weight influencing principle to prevent adrenal atrophy. Since the general tendency is to produce more highly purified preparations of corticotrophin this point may become increasingly important in the future.

CONCLUSIONS

There are at least two factors of pituitary origin which have effects on the adrenal cortex. It is likely that one factor merely potentiates the growth promoting effects of the other factor.

From the data collected during investigations subsidiary to the study of the effects of corticotrophin on human adrenal cortex it appears that :-

(a) the adrenal weight is considerably greater after death (6 g.), than during life (4 g.).

(b) the mean weight of the right human adrenal approximates closely to the weight of the left human adrenal.

(c) the sex difference in adrenal weights noted by others was not observed here.

S E C T I O N II

EFFECT OF CORTICOTROPHIN ADMINISTERED IN VIVO ON THE NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOSPHATE LINKED DEHYDROGENASES IN THE ADRENAL CORTEX

The unusually high glucose-6-phosphate dehydrogenase (G6PD) activity found in the adrenal cortex of the ox and the rat (Kelly et al., 1955) and the fact that this enzyme activity is linked with the production of the reduced form of the nicotinamide-adenine dinucleotide phosphate ($NADPH_2$), which is required for corticosteroid hormone biosynthesis (Fig. 13), prompted an investigation of G6PD and the other principal NADP reducing enzymes in the adrenal cortex of man, and the effect of corticotrophin on these enzymes.

Since corticotrophin stimulates adrenal growth as well as steroidogenesis, the enzyme activities were assayed in adrenal cortex stimulated by corticotrophin of low growth promoting potency, as well as in the adrenal cortex stimulated by cruder preparations of corticotrophin. The time factor in the enzyme changes observed, and the distribution of these enzymes within the cell and within the adrenal cortex were also studied.

The principal observations were made on the human adrenal cortex, but some of the experiments were repeated on rats and guinea pigs.

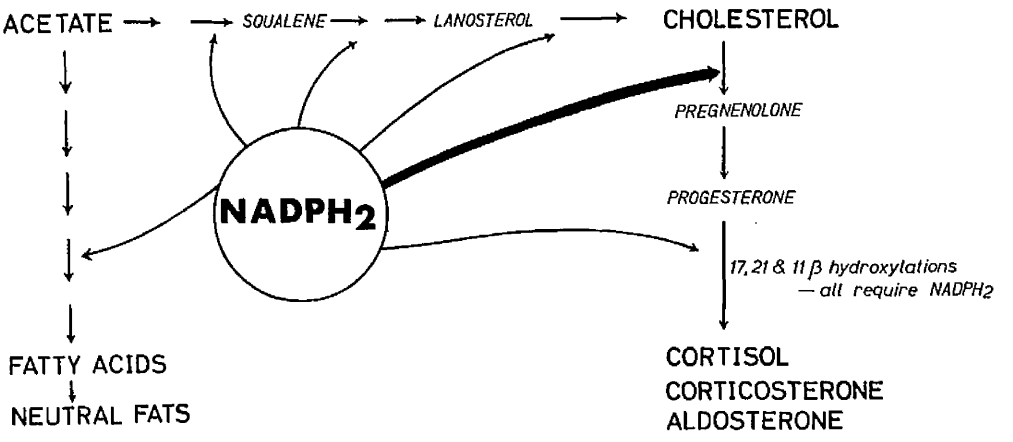


Fig. 13. The importance of the reduced coenzyme $NADPH_2$ for biosyntheses of corticosteroid hormones and lipids in general.

MATERIALS AND METHODS

The adrenal glands used in the experiments described in this section were:-

1. Glands from adult female patients with advanced breast carcinoma undergoing two stage bilateral adrenalectomy. This was the principal group studied here and the procedure and hormone administration is described on page 40 in Section I.

2. Glands from a similar group of patients who were undergoing one stage bilateral adrenalectomy. Two different experiments were performed in this group.

(a) Hormones were not administered before the operation, but immediately after one adrenal was removed an intravenous infusion of corticotrophin was started. 75 I.U. of lyophilised ACTH-AR were given in one pint of isotonic saline, during a period of time which varied from 30-90 minutes.

(b) ACTH was administered intramuscularly before the operation. 40 I.U. were given at each injection; the injections were given 8 hourly in experiments lasting 42 hours or less, and 12 hourly in experiments of longer duration.

3. Rat and guinea pig adrenals were obtained as described on page 42 in Section I.

Tissue preparations.

All adrenals were removed with minimum of handling, immediately

placed on crushed ice, and all subsequent operations were carried out near 0°C. Enzyme assays were completed within 8 hours of removal of the glands. In the case of the human glands, medulla and connective tissue capsule were separated from the cortex and discarded; animal adrenals were expressed from the capsule and used whole. The tissue was then treated as follows:-

(a) A 10% (w/v) homogenate was prepared in 0.15 M KCl in a Potter homogeniser having a nylon pestle and uniform bore glass tube. The homogenate was centrifuged for 30 minutes at 15000 g. and 0°C. The supernatant liquid was used for dehydrogenase assays.

(b) The intracellular distribution of enzymes was studied using a 10% (w/v) homogenate prepared in 0.25 sucrose solution containing 0.12 M nicotinamide. The cell fractions were separated according to the method of Schneider (1948).

(c) In order to study the distribution of enzyme activity throughout the adrenal cortex, thin flat pieces of the cortex approximately 1 cm. square were frozen on a microtome chuck with capsule next to the chuck. Serial sections were cut in the cryostat from the medullary border of the zona reticularis outwards. The thickness of the sections taken for enzyme study was 100 μ, and intervening 10 μ sections were taken for histology. The weight of each 100 μ section (about 10 mg.) was quickly determined by a micro torsion balance and the slice transferred to a 8 x 50 mm. cellulose acetate tube containing

0.15 M KCl at 0°C. A 5% (w/v) homogenate was prepared by exposure to ultra sound for 20 seconds (Mullard Ultra Sonic Disintegrator, M.S.E., London) and the homogenate was transferred to a microcentrifuge tube. The filled tubes were centrifuged for 30 minutes at 15000 g and 0°C. The supernatant was used for dehydrogenase assays and nitrogen determinations.

Enzyme assays.

Isocitric dehydrogenase (ICD) and malic enzyme (ME) were assayed by the method Ochoa (1955).

G6PD and 6-phosphogluconate dehydrogenase (6PGD) were assayed by the procedure outlined by Glock and McLean (1953). Overnight dialysis was omitted, as it was found that G6PD is very unstable and such a procedure results in a considerable and variable loss of activity. In its place enzyme preparations containing NADP were kept for several minutes at room temperature to allow NADP reduction by endogenous substrates to take place.

Lactic Dehydrogenase (LD) was assayed by the method of Molanda (1955).

Phosphohexose isomerase (PHI) was measured by a reaction linked to G6PD activity, as described by Stein (1955).

A 0.05 M glycylglycine-NaOH buffer at pH 7.6 was used in all assays. The temperature of the reaction mixtures was 20-22°C. All the assays depend on that property of the enzymes which is also their

principal physiological role, namely, the ability to cause reduction of the nicotinamide adenine dinucleotides in presence of the appropriate substrate. This reduction causes an increase in the optical density (O.D.) of the reaction mixture at 340 mμ. The O.D. was measured in 0.5 ml. cells of 10 mm. light path using the Unicam SP 500 Spectrophotometer. Readings were taken against a "blank" cell containing all components of the reaction except the appropriate nucleotide.

All enzyme activities are expressed in arbitrary units per gram wet weight of adrenal cortex or per 10 mg. of nitrogen in the enzyme preparation. One unit is the amount of enzyme which will reduce 0.01 μ mole of NADP per minute. G6PD activity in the human adrenal cortex is expressed as the rate of reduction of NADP on addition of G6P, without any allowance being made for the subsequent reduction of NADP by 6PG, which is the product of G6P oxidation. This is permissible in the case of the human adrenal cortex, since the activity of 6PGD is low in comparison with G6PD and the error introduced is much smaller than that of the assay as a whole. In the rat and guinea pig the two substrate method of Glock and Nelson (1953) was employed for the assay of G6PD. In this method both G6P and 6PG are added to the cuvette and the activity obtained with 6PG alone is subtracted from the resulting value.

Conditions of Enzyme Assay.

(a) Buffer, pH, cations.

There is no information in the literature on the properties of the enzymes present in the human adrenal cortex. It was necessary, therefore, to study some of the properties of the enzymes under investigation in order to establish reliable assay conditions. The properties of the enzymes G6PD and 6PGD of rat liver were described in detail by Glock and McLean (1953), and the descriptions of the enzymes reported by them were found to apply also to the enzymes present in the human adrenal cortex. For instance, the dependence of activity on pH, depicted in Fig. 14, is like that of the liver enzymes. Similarly, divalent cations are necessary for optimum enzyme activity; Mg^{++} was found to be best for G6PD and 6PGD activity, and Mn^{++} for ICD and ME. Glycylglycine was a better buffer than veronal for G6PD and 6PGD (Table XI).

(b) Linearity of assay.

Increasing the concentration of the cofactors (NADP or Mg^{++}, Mn^{++}) or of the substrate of the enzyme action had no effect on the reaction rate, but there was good linearity between the amount of the monogonate added and enzyme activity calculated. Table XII shows this for all the principal enzymes studied here.

(c) Activity of the enzymes under study in other constituents of the adrenal gland.

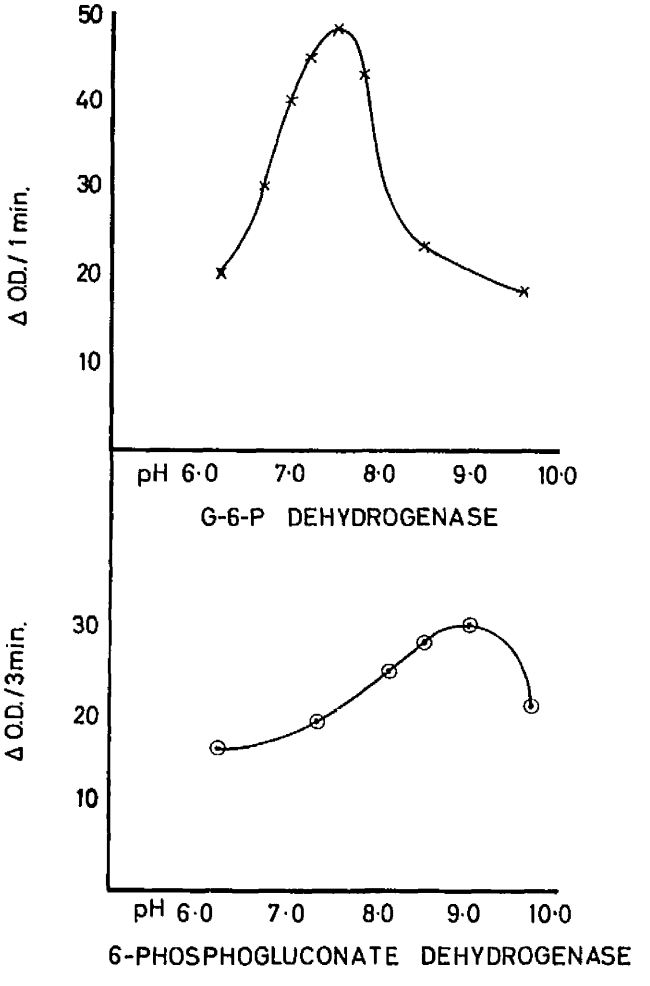


Fig. 14. Dependence of enzyme activity on pH for G6PD and 6PGD.

TABLE XI

Comparison of activities of the principal NADP linked dehydrogenases of the human adrenal cortex in different buffers

	Veronal buffer	Glycylglycine buffer
G6PD	288	432
6PGD	15	20
ICD	648	672
ME	10	10

The pH of the buffer was in each case 7.6

TABLE XII

Proportionality of the change in optical density (O.D.) to the amount of homogenate added

Amount of homogenate	Change in O.D. per minute			
	ICD	G6PD	6PGD	ME
0.02 ml.	35	14	-	-
0.05 ml.	82	29	-	-
0.10 ml.	160	62	8	5
0.15 ml.	233	101	14	9
0.20 ml.	-	130	18	14

Many adrenal glands studied here were markedly congested or haemorrhagic, especially those obtained after corticotrophin administration to the patient. If blood contains a high level of NADP reducing enzymes any alteration in activity could be due to the higher blood content of the adrenal. Estimations were therefore carried out to determine G6PD activity, since this is the enzyme showing principal changes after corticotrophin administration.(Table XIII). The mean value of four determinations of G6PD was 5.8 units per ml. of packed erythrocytes, and plasma was without any activity. It is clear that since the activity in the blood is 50 times less than in the adrenal cortex (cf. Table XIII), changes in blood content of the gland will have no appreciable effect on G6PD of the homogenate.

Similarly, contamination of the enzyme preparation with portions of the adrenal medulla might influence results if activity of the enzymes in the medulla exceeds the activity in the cortex. All the enzymes studied here, however, have lower activity in the medulla.

Nitrogen determinations were performed on enzyme preparations by the method described by Le Page (1957). It is based on digestion of the homogenate supernatant with sulphuric acid containing a mercury catalyst followed by direct Nesslerization. The colour was read in the SP 500 spectrophotometer at 425 mμ.

Hormone Preparations.

Corticotrophin preparations and cortisone used in the experiments

described in this section were of the types described on pages 43 and 44 in Section I.

Reagents.

Glucose-6-phosphate disodium salt (G6P), 6-phosphogluconic acid sodium salt (6PG), fructose-6-phosphate barium salt (F6P) (this was converted to the sodium salt by addition of an equivalent amount of Na_2SO_4 in solution), nicotinamide-adenine dinucleotide (NAD) and nicotinamide-adenine dinucleotide phosphate (NADP) were obtained from Sigma Chemical Company (U.S.A.), isocitrate trisodium salt from the California Foundation for Biochemical Research (U.S.A.), L-malic acid from the Nutritional Biochemicals Corporation (U.S.A.).

RESULTS

1. Effect of Corticotrophin on the NADP Linked Dehydrogenases of the Human Adrenal Cortex.

(a) Corticotrophin administered to patients undergoing two stage bilateral adrenalectomy.

Table XIII shows the enzyme activities in the homogenates of adrenals of patients who were given commercial preparations of corticotrophin. It is seen that there is a marked increase in G6PD activity (approximately +100%). This increase is statistically highly significant, but the slight alterations in the activity of the other enzyme studies (ICD and 6PGD) are of no significance.

It has already been shown (page 66 and Table V) that commercial preparations of corticotrophin induce a considerable increase in adrenal weight. It was of interest to investigate whether the observed increase in G6PD activity is seen when adrenal growth stimulation is minimal, as after administration of ACTH-A_1A_2 (defined on page 20). The enzyme activities obtained after ACTH-A_1A_2 administration are shown in Table XIV, where it may be seen that the results are essentially similar to those seen after the administration of commercial ACTH. In order to study further the effect of adrenal growth, enzyme activities were assayed in glands stimulated *in vivo* with crude ACTH (Table IV), which has high growth promoting activity (see page 46) Again, enzyme alterations are similar to those seen in the other groups, i.e., G6PD increases markedly, while 6PG and ICD show no statistically

TABLE XIII

Effect of commercial preparations of long acting corticotrophin on the principal NADP reducing enzymes of the human adrenal cortex

	Enzyme activities (means of 4 cases) ± S.D.		
	G6PD	6PG	ICD
First gland - no ACTH	337 ± 107	108 ± 39	612 ± 101
Second gland - ACTH pretreatment	643 ± 85	132 ± 40	682 ± 14
Difference	306	24	70
Probability that this difference if significant	< 0.001	> 0.05	> 0.05

Enzyme activity in arbitrary units (defined on page 68) per gram wet weight. 40 I.U. ACTH-AR gel given every 12 hours starting 100 hours before the second operation. Individual differences within each pair of glands from any one patient were used to calculate the significance of the difference of means.

TABLE XIV

Effect of ACTH-A_1A_2 on the NADP reducing enzymes of the human adrenal cortex

	Mean adrenal weight	Enzyme activity (mean of 5 cases) ± S.D.			
		G6PD	6PGD	ICD	ME
First gland - no pretreatment	4.0 g.	318 ± 78	85 ± 32	929 ± 158	41 ± 18
Second gland - ACTH pretreatment	4.8 g.	714 ± 92	118 ± 44	1088 ± 128	43 ± 27
Difference	0.8 g.	396	33	159	2
Probability that the difference is significant		<0.001	0.05-0.02	>0.05	>0.05

Enzyme activity in arbitrary units (page 68) per gram wet weight. 40 I.U. ACTH-A_1A_2 were given every 12 hours starting 100 hours before the second operation. Individual differences within each pair of glands from any one patient were used to calculate the significance of the difference of means.

TABLE XV

Effect of crude ACTH on the NADP reducing enzymes of the human adrenal cortex

	Mean adrenal weight	Enzyme activity (mean of 5 cases) ± S.D.			
		G6PD	6PGD	ICD	ME
First gland - no pretreatment	4.0 g.	277 ± 118	69 ± 41	661 ± 90	37 ± 17
Second gland - ACTH pretreatment	8.1 g.	699 ± 260	105 ± 91	769 ± 183	64 ± 32
Difference	4.1 g.	422	36	108	27
Probability that the difference is significant		< 0.01	> 0.05	> 0.05	< 0.05

Enzyme activity in arbitrary units (page 68) per gram wet weight. 40 I.U. of crude ACTH were given every 12 hours starting 100 hours before the second operation. Individual differences within each pair of glands from any one patient were used to calculate the significance of the difference of means.

significant increase. ME shows an increase when crude ACTH was given, but the estimations of ME were less accurate and are therefore less reliable than the estimations of the other enzymes.

Tables XIII, XIV and XV are based on enzyme activity per unit wet weight of adrenal tissue. Since, however, the nitrogen content of a unit wet weight increases after corticotrophin administration (Table XVI), the enzyme activities were recalculated in terms of cell nitrogen content. The results showed that the changes in enzyme activity of the adrenal cortex were not altered by this method of expressing the results.

In the group where adrenals were removed in exactly the same two stage procedure but cortisone instead of corticotrophin was administered before the removal of the second gland, there is no significant change in any enzyme activity (Table XVII).

Lactic dehydrogenase is also capable of reducing NADP though it has much greater affinity for NAD. The activity of this enzyme was therefore assayed before and after corticotrophin, and the results presented in Table XVIII show that corticotrophin causes no alteration in the activity of this enzyme.

(b) Corticotrophin administered to patients undergoing one stage bilateral adrenalectomy.

(i) Short term experiments.

Corticotrophin (commercial preparations) was administered

TABLE XVI

Nitrogen content of supernatant fraction obtained by centrifuging at 15,000 g. for 30 min., 10% (w/v) homogenates of the human adrenal cortex prepared in 0.15M KCl solution

	1st operation	2nd operation	1st operation	2nd operation
Hormone administered	None	ACTH-A_1A_2	None	Crude ACTH
Number of cases	5	5	5	5
Mean total N. content (mg%)	136	169	150	165
Mean increase after ACTH ± S.D.	33 ± 18		15 ± 10	

Each preparation fo corticotrophin was administered in the same way. 40 I.U. were given 12 hourly for 100 hours before the operation.

TABLE XVII

Comparison of enzyme activities in human adrenal cortex when corticotrophin was not administered before either operation

	Mean enzyme activity (4 cases) ± S.D.			
	G6PD	6PGD	ICD	ME
First side	299 ± 42	62 ± 36	820 ± 82	25 ± 22
Second side	308 ± 50	55 ± 24	770 ± 96	20 ± 11
Change	+9	-7	-50	-5
Significance	P > 0.05	P > 0.05	P > 0.05	P > 0.05

Enzyme activity is expressed in arbitrary units (page 68) per gram wet weight. 50-100 mg. of cortisone were given daily in the interval between the two operations. The significance has been calculated on the basis of individual differences within each pair of glands from the same patient.

TABLE XVIII

Effect of corticotrophin on the lactic dehydrogenase of the human adrenal cortex

Activity of L.D. in 9 adrenals	Not pretreated with ACTH	ACTH treated (crude and A_1A_2)
Mean	2100	2000
S.D.	± 350	± 420

Enzyme activity is expressed in arbitrary units similar to those defined on page 68 per gram wet weight, but to facilitate assay NAD was used instead of NADP. 40 I.U. of crude ACTH or commercial corticotrophin were given every 12 hours starting 100 hours before the second operation.

by intravenous infusion, as described on page 65. Table XIX shows that no alteration in activity of either G6PD or ICD can be detected in periods of time up to 90 minutes.

(ii) Experiments of several days duration.

The effects on NADP reducing dehydrogenases of intramuscular administration of crude ACTH or commercial ACTH to patients not previously adrenalectomised are shown in Table XX and the values are compared with those obtained for glands removed without any hormone pretreatment. From this it may be seen that corticotrophin appears to increase the activity of G6PD, and this increase becomes apparent within 20 hours of starting corticotrophin administration. The increase in G6PD activity is, however, less marked than in those subjects who had previously undergone the first stage of two stage bilateral adrenalectomy.

2. Distribution of the Enzymes Studied.

(a) Intracellular distribution.

Under the conditions of assay employed here it was possible to study the intracellular distribution only in the case of the most active enzymes, namely ICD and G6PD. In the case of the less active 6PGD and ME, light scattering effects associated with the use of preparations containing whole cells, nuclei and mitochondria, interfered seriously with the measurement of small changes in optical density. G6PD activity of the whole homogenate was recovered completely in

TABLE XIX

Effect on NADP linked dehydrogenase activity of commercial ACTH administered intravenously (i.v.) for short periods

Patient	Period of intravenous administration of corticotrophin	Gland removed	Units of Enzyme Activity in adrenal cortex	
			ICD	G6PD
1.	30 minutes	1st	584	300
		2nd	577	269
2.	45 minutes	1st	762	288
		2nd	742	301
3.	90 minutes	1st	672	432
		2nd	648	468

Enzyme activity is expressed in units (page 68) per gram wet weight. 75 units of lyophilized ACTH-AR were administered in one pint of isotonic saline intravenously, over the period indicated, starting immediately after the removal of the first adrenal gland.

TABLE XX

Effect of NADP linked dehydrogenase activity of ACTH administered to patients not previously adrenalectomised

Patient	Gland	Period of ACTH administration before operation (hours)	Total amount of corticotrophin administered (i.u.)	Units of enzyme activity in adrenal cortex			
				G6PD	6PGD	ICD	ME
1.	Right	20	225	530	43	549	48
	Left			467	65	561	47
2.	Right	42	240	530	194	629	52
	Left			603	217	723	58
3.	Right	96	320	395	82	700	48
	Left			531	116	940	31
4.	Right	96	320	578	141	701	75
Average for 20 patients not previously treated with ACTH and S.D.				316 ± 84	80 ± 37	782 ± 203	41 ± 13

Enzyme activity is expressed in units (page 68) per gram wet weight. 40 I.U. of crude ACTH or ACTH-AR gel were administered intramuscularly at intervals of 8 or 12 hours over the period indicated in each case.

the soluble part of the cell sap. IDD activity was found mainly in the same location as is summarised in Table XXI. The only measureable activity of G6PD and ME was found in the soluble part of the cell sap.

Mitochondrial preparations were exposed to ultrasound for periods up to 5 minutes, were repeatedly frozen and thawed, or were suspended in hypotonic media in an attempt to destroy membranes which enclose the mitochondria and which may limit the access of substrates to the mitochondrial enzymes. Such treatment increased the activity of the mitochondrial IDD to approximately 10% of the total cell activity, but activity of the other enzymes did not appear. An example is shown in Table XXII.

(b) Distribution of G6PD within the adrenal cortex.

The distribution of G6PD activity throughout the adrenal cortex was observed in tissue sections 100 μ thick cut parallel to the capsule. In glands removed before corticotrophin administration the enzyme activity is distributed more or less uniformly in all zones of the cortex. However, following corticotrophin treatment of the patient the increase in G6PD activity takes place mainly in the middle third of the cortex, with a peak of activity in the region which corresponds to the border between the compact and the clear cells (Fig. 15). The position of this junction was established by histological examination of the gland as a whole (see Fig. 16) and also of 10 μ sections removed between each 100 μ section taken for enzyme assay. Fig. 16 shows that

TABLE XXI

Intracellular distribution of ICD and G6PD

in human adrenal cortex

	Nuclei	Mitochondria	Microsomes	Supernatant
ICD	0	3%	0	100%
G6P	0	0	0	100

TABLE XXII

Effect on NADP linked dehydrogenases of homogenising adrenocortical tissue in distilled water and isotonic KCl

	ICD	G6PD	6PGD	ME
Homogenate in KCl	425	154	29	24
Homogenate in H_2O	468	145	29	26
Increase in activity	10%	none	none	none

Enzyme activities are expressed in units (page 68) per gram wet weight of the starting material. The homogenate was 10% (w/v) in each case.

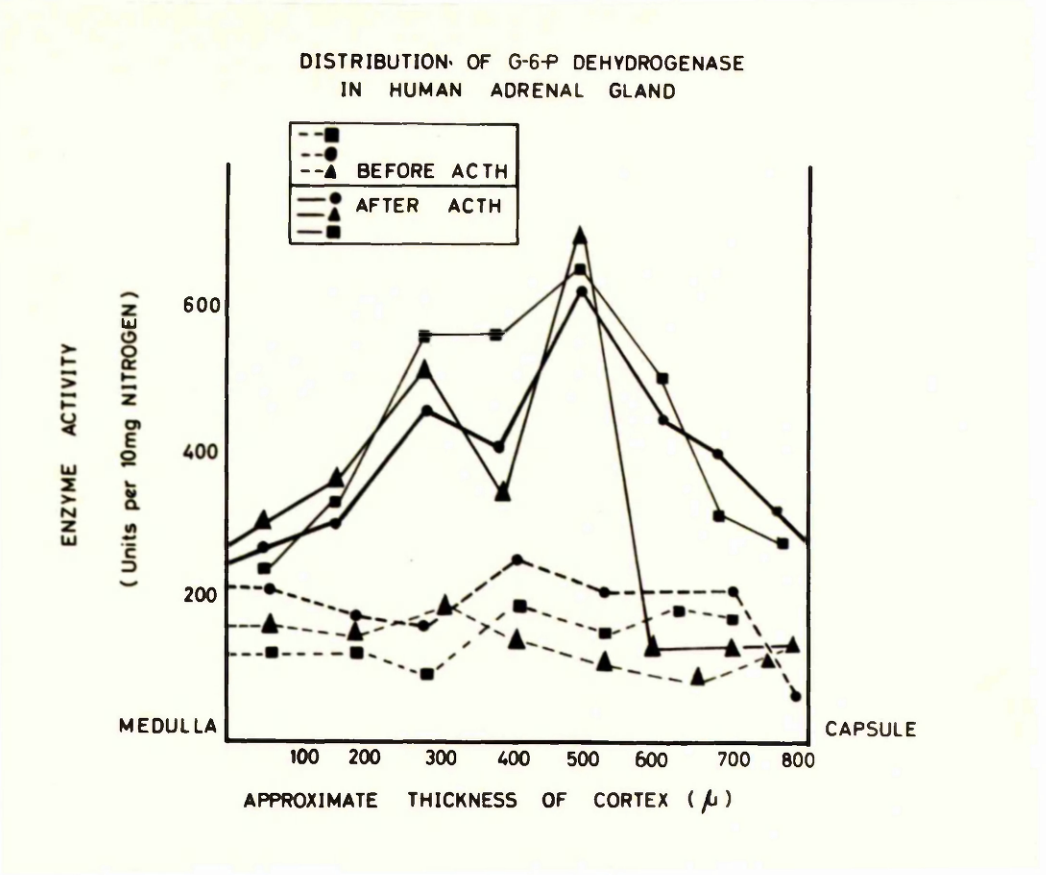


Fig. 15.

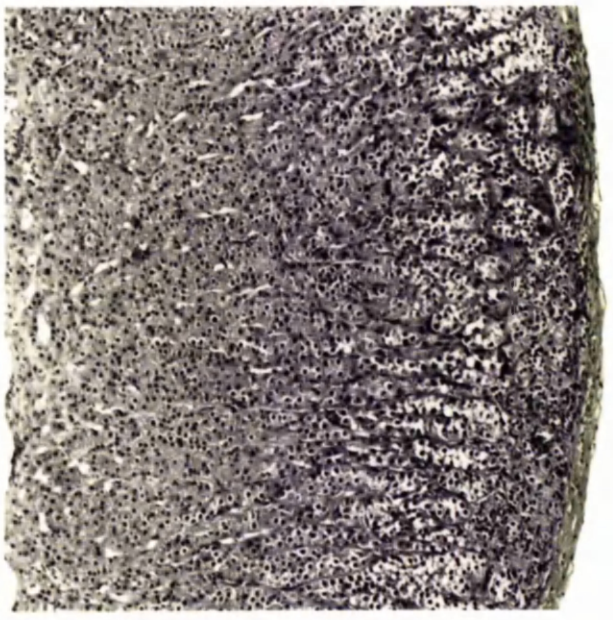


Fig. 16.

Figs. 15 and 16. The upper figure shows the distribution of G6PD activity in six different adrenal glands. The lower figure shows the histological appearance of one of the glands after ACTH (commercial preparation). Comparison of the two figures shows that peak activity corresponds to the border between the clear and the compact cells. Lower Fig. x 60.

the region which has the highest G6PD increase as shown in Fig. 15, is the region of the cortex which in a normal unstressed gland consists of clear cells, but now contains compact cells.

3. The Effect of 6PG on Phosphohexose Isomerase activity.

The increase in G6PD activity after corticotrophin administration is not paralleled by a similar increase in 6PGD activity. This should result in an increased concentration of 6PG in the adrenocortical cells. Parr (1956) has shown for some mammalian tissues that 6PG inhibits phosphohexose isomerase (PHI), the enzyme which catalyses the entry of G6P into the glycolytic pathway. If this also holds for the adrenal cortex in man, the blocking of PHI by 6PG would tend to increase the proportion of G6P metabolised in the pentose phosphate pathway, at the expense of the glycolytic pathway. The data presented in Table XXIII show that 6PG does in fact markedly inhibit PHI *in vitro*, but that there is no significant inhibition of G6PD by 6PG.

4. Effect of Corticotrophin on NADP Linked Dehydrogenases in the Adrenals of Laboratory Animals.

(a) Rats.

Table IX in Section I shows that in the rat the administration of crude ACTH causes a greater increase in adrenal weight than the administration of ACTH-A_1A_2. Table XXIV shows the effect of these preparations on the NADP reducing enzymes. It is evident that both types of corticotrophin used cause marked increase of the pentose

TABLE XXIII

Inhibition of phosphohexose isomerase by 6 phosphogluconic acid

Substrate	Rate of NADP reduction in "units"
5 µmoles F6P	233
5 µmoles 6PG	19
5 µmoles F6P + 5 µmoles 6PG	96
i.e., reduction of TPN due to F6P in presence of 6PG	77
5 µmoles G6P	405
5 µmoles G6P + 5 µmoles 6PG	410

The system consisted of 130 µmoles of glycylglycine, 60 µmoles Mg^{++}, 0.2 µmoles TPN and 0.1 ml. of adrenal cortex supernatant in total volume of 3 ml., plus indicated additions. Enzyme units are defined on page 68.

TABLE XXIV

Effects of ACTH-A_1A_2 and crude ACTH on the enzymes of the rat adrenal gland

	Number of animals	Mean enzyme activity ± S.D.			
		G6PD	6PGD	IDD	MD
Non injected controls	4	685 ± 198	460 ± 190	300 ± 107	
Injected controls	8	715 ± 154	540 ± 69	350 ± 113	100 ± 29
ACTH-A_1A_2	8	1320 ± 211	1060 ± 267	690 ± 73	227 ± 89
Crude ACTH	8	1120 ± 151	1056 ± 89	330 ± 93	150 ± 29

Enzyme activity is expressed in units (page 68) per gram wet weight. 4 I.U. of corticotrophin were given intramuscularly to each rat twice daily for 4 days.

phosphate cycle enzymes (G6PD and 6PGD), but that 6PGD increases rather more markedly. In addition, however, the purified preparation ACTH-A_1A_2 causes a significant increase in the two members of Krebs' cycle, ICD and ME.

(b) Guinea pigs.

Table X in Section I shows that the administration of crude ACTH to guinea pigs causes more marked stimulation of adrenal growth than the administration of highly purified ACTH-A_1A_2. The enzyme activities in these groups of glands are shown in Table XXV. It is clear that corticotrophin causes significant increases of G6PD, 6PGD and ME but there is no alteration in ICD activity. The increase in 6PGD is the most marked and is of the same magnitude after ACTH-A_1A_2 as after crude ACTH. The other enzymes, however, (i.e., G6PD and ME) show a greater increase in activity after crude ACTH, when more intense growth is taking place. This is illustrated in Fig. 17 where it can be seen that G6PD and ME show an increase in activity which is proportional to the increase in adrenal weight. 6PGD, on the other hand, shows a marked increase independent of the rate of growth.

The time factor in the changes in activity of the enzymes showing the principal increases after corticotrophin is shown in Fig. 18. Corticotrophin was administered to a group of 5 guinea pigs for varying periods of time and the enzyme activities in the adrenal gland were then assayed. It is seen that the increase in G6PD activity has a

peak at an early stage of corticotrophin administration and then becomes progressively less pronounced, while G6PD activity remains elevated at the same relatively high level during corticotrophin administration.

TABLE XXV

Effects of ACTH-A_1A_2 and crude ACTH on the enzymes of the guinea pig adrenal gland

	Number of animals	Mean enzyme activity ± S.D.			
		G6PD	6PGD	ICD	ME
Controls (injected with saline)	6	209 ± 34	60 ± 12	1214 ± 160	38 ± 7.2
ACTH-A_1A_2	6	340 ± 42	132 ± 25	1269 ± 152	52 ± 15.5
Crude ACTH	6	467 ± 49	124 ± 13	1163 ± 189	63 ± 8

Enzyme activity is expressed in units (page 68) per 10 mg. nitrogen: 4 I.U. of corticotrophin were given intramuscularly to each animal twice daily for 4 days.

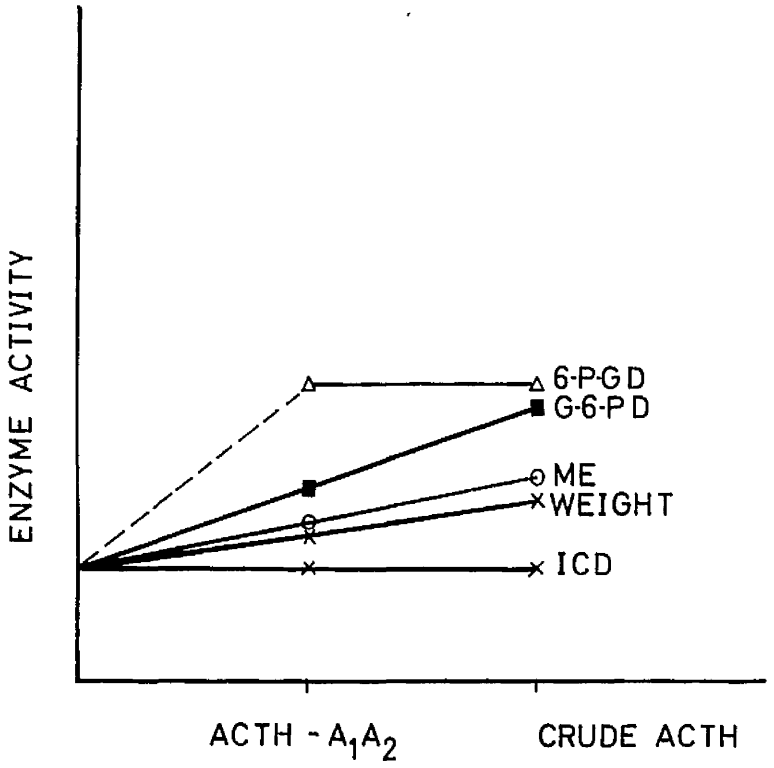


Fig. 17. Relative increases in adrenal weight and four NADP linked dehydrogenases in the adrenal gland after administration of corticotrophin preparations of differing purity to guinea pigs. Arbitrary scale. Only 6PGD increases to the same extent after administration of either preparation.

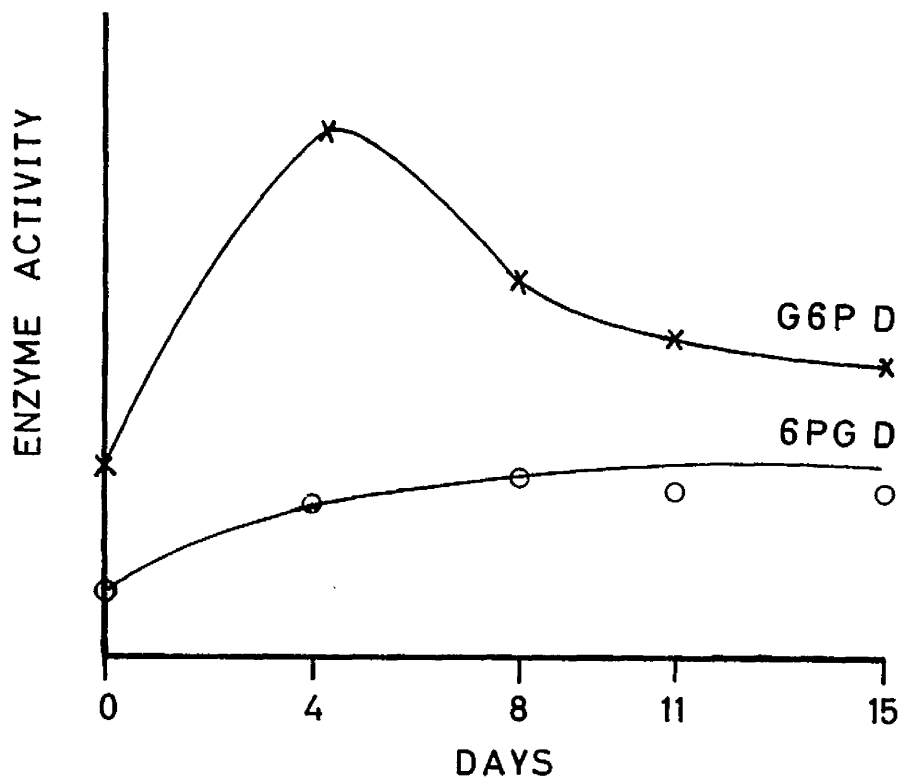


Fig. 18. Activities of G6PD and 6PGD in the guinea pig adrenal after varying times of administration of corticotrophin (crude) to the animal.

DISCUSSION

1. Metabolic Role of the Enzymes Studied.

The principal metabolic pathways in carbohydrate metabolism are shown in Fig. 19, and the position of the enzymes studied are indicated in this scheme. G6PD and 6PGD are the first two enzymes concerned in the operation of a cyclic process known as the Hexose-Monophosphate Shunt or the Pentose Phosphate Cycle. ICD is an important member of the tricarboxylic acid or Krebs' Cycle, and ME catalyses an alternative route within this cycle. It is important to note that G6PD acts on a substrate which can be metabolised along several alternative pathways. Thus its activity may well be a critical factor in channeling the substrate, G6P into the pentose phosphate cycle. The product of its action, 6-phosphogluconic acid, is the substrate for another NADP linked dehydrogenase, 6PGD. The product of this reaction is ribulose-5-phosphate, a 5-carbon phosphorylated sugar which is one of the starting materials for the synthesis of nucleic acids.

The reduced co-enzyme, $NADPH_2$, is a key cofactor in many biosynthetic processes of the cell. It is necessary for the biosynthesis of lipids (e.g., for the action of crotonyl CoA reductase) and proteins (e.g., for glutathione reductase) and since these are the principal components of the structural elements of the cell, $NADPH_2$ can be expected to be essential for cell growth. But $NADPH_2$ is also particularly important in the biosynthesis of steroids, as it has now been

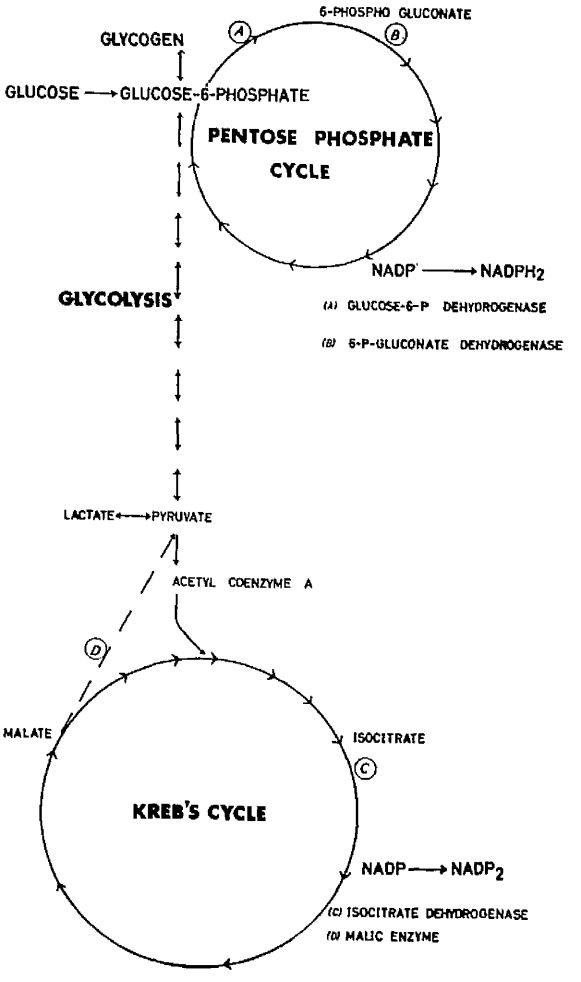


Fig. 19. An outline of the principal pathways of carbohydrate metabolism. The principal NADP linked dehydrogenases catalyse the reactions indicated by circled letters.

demonstrated to be essential for at least seven steps in the formation of cortisol from acetate (Fig. 13). In addition to the well known requirement for $NADPH_2$ for the hydroxylation of the steroid molecule in the 11β, 21 and 17α positions (see Grant, 1960), in the cyclisation of squalene (Goodman, 1961) and in the conversion of lanosterol to cholesterol (Olson et al., 1957), it has recently been shown that $NADPH_2$ is essential for the reduction of 3-hydroxy-3-methyl glutamic acid to mevalonic acid (Durr and Rudney, 1960) and for the side chain cleavage of cholesterol in its conversion to pregnenolone (Haikerston et al., 1961).

The conversion of cholesterol to pregnenolone is probably the step in the adrenocorticoid hormone production which is accelerated by corticotrophin (Stone and Hechter, 1954). The demonstration that $NADPH_2$ is required for this reaction fits in well with the theory of Haynes (Fig. 7) that the action of corticotrophin is mediated by the increase in the availability of this cofactor. This specific role of $NADPH_2$ in the mechanism of corticotrophin action is also supported by the work of Koritz and Peron (1958) who showed that $NADPH_2$ has the same action as corticotrophin on the stimulation of corticoid production by adrenal slices *in vitro*.

2. The Significance of the Increases in Enzyme Activity.

The experiments presented in this section show the effect of corticotrophin on the dehydrogenases of the pentose phosphate cycle

and some Krebs' cycle enzymes and shows that there is a species difference in the response. In man G6PD alone shows an increase in activity (Table XIII, XIV and XV) while in rat and guinea pig 6PGD shows more marked increase than G6PD (Tables XXIV and XXV).

The increase in G6PD and 6PGD activity observed in the adrenal cortex after corticotrophin is not unique to this situation. Other workers found that the activity of these enzymes can vary under different conditions both in mammalian tissues and in bacteria. Glock and McLean (1954) found an increase of G6PD and 6PGD in the rat mammary gland during lactation. The same authors (1955) reported an increase in these enzymes in rat liver after insulin and thyroxine (Glock, McLean and Whitehead, 1956), and the latter finding was confirmed by Huggins and Yao (1959). Marked increases in G6PD activity were found in livers of rats on a high carbohydrate diet (Tepperman and Tepperman, 1958), and Weber and Cantero (1957) noted that G6PD is five times as active in Novikoff hepatoma as in normal rat liver. It has also been found that the activities of G6PD and 6PGD in rat uterus are markedly enhanced by administration of oestradiol-17 β (Scott and Lisi, 1960), but that the activity of ICD remains constant after such treatment (Mongkolkul and Grant, 1960). In bacteria similar effects can be seen, e.g., treatment of E. coli with 2:4 dinitrophenol (Scott, 1956) results in an increase in G6PD.

Thus the enzymes G6PD and 6PG seem to be responsive in the

mammal as well as bacteria to changes in nutrition, to changes in hormone balance and to changes associated with neoplasia.

The demonstration that G6PD increases in the human adrenal cortex after corticotrophin administration can be regarded as another example of mammalian enzyme adaptation. This phenomenon has been defined as alteration in the quantity of an active enzyme in the cells of the animal in response to chemical stimuli (Knox, 1962). In each cell the genes determine what particular kind of protein is synthesized, and these proteins have structural or enzyme properties. Quantitative modifications of the enzyme pattern can however occur in response to chemical substances present in the cell and the enzyme activity increases when a substrate or some other suitable chemical stimulus arrives in the cell. This increase in activity may be due to synthesis of the enzyme from the individual amino acids, or to a conversion of a large inactive precursor. There is no evidence as to which of these processes takes place in the adrenal cortex when G6PD activity increases after corticotrophin stimulation.

Other enzymes have been found to increase after corticotrophin administration. An increase in phosphorylase in activity has been observed by Haynes and Berthet (1957) in an adrenal slices. Grant et al. (1957) showed that 11β-hydroxylation of deoxycorticosterone is increased in human adrenal cortex following corticotrophin administration. More recently nucleotide metabolising enzymes have also been found to

increase after corticotrophin (Hill et al., 1961).

The increase in G6PD and 6PGD activity in the mammalian adrenal cortex found here will result in a larger potential for the production of $NADPH_2$. This coenzyme is generally regarded as of greater importance for biosynthetic reactions than for energy production (Ernster and Navazio, 1957). In the adrenal cortex $NADPH_2$ may be necessary both for growth processes and for corticosteroid biosynthesis (Fig. 13); similarly the work on G6PD in other tissues reviewed on page 81 suggests that this enzyme increases when the tissue is growing (hepatoma, growing uterus) or when secretory processes are taking place (lactating mammary gland). The use, therefore, of corticotrophin preparations of different adrenal growth promoting activity was an attempt to separate those consequences of corticotrophin action which are due to adrenal growth, from those which are concerned with steroid biosynthesis. Since all preparations of corticotrophin used in the experiments reported here caused some weight increase, a clear cut separation of the effects was not achieved. Nevertheless, the intensity of growth stimulation varied considerably; the weight increase of the human adrenal after crude ACTH was on the average five times as marked as after ACTH-A_1A_2 (Tables VI and VII) yet the increase in G6PD was approximately the same. Corticotrophin was administered in the same dosage to each group; although the dosage is based on ascorbic acid depletion, there is good evidence that this corresponds to steroidogenic

potency of the preparation (see page 16). It would seem therefore that the increase in G6PD activity noted in the human adrenal cortex after corticotrophin stimulation *in vivo* is related principally to the requirement for increased corticosteroid biogenesis, and is not merely a consequence of accelerated adrenal growth.

In the rat and guinea pig adrenal cortex the situation is analogous to the extent that the pentose phosphate cycle dehydrogenases increase markedly after corticotrophin stimulation, but the dehydrogenases associated with Krebs' cycle show less marked increase unless in rats treated with A_1A_2 (Table XXIV). It is evident in Tables XXIV and XXV that the principal increase is seen in both 6PGD and G6PD activity. The exact significance in the changes in enzyme pattern after corticotrophin in the rat adrenal are difficult to interpret but a possible explanation is that when growth is taking place very actively, certain enzymes and proteins are needed more urgently than the enzymes of Krebs' Cycle. When growth is less intense, as after ACTH-A_1A_2 administration, the resources of the cell can be utilised to form other enzymes including those which facilitate steroid biosynthesis. Thus, when crude ACTH is given, ICD and ME show no increase, but administration of ACTH-A_1A_2 resulted in marked increase in activity of these enzymes. As regards the relative increase in the dehydrogenases of pentose phosphate cycle, 6PGD shows more marked increase than G6PD, and this is also evident in the work reported by other authors.

Huggins and Yao (1959) found that these enzymes increase in the rat liver after various hormonal treatments, but while 6PGD could increase without any changes in G6PD activity, G6PD increase was observed only if 6PGD showed an increase. Rudolph and Olson (1955) found that hypophysectomy results in a marked decrease of 6PGD activity in rat adrenals but has no effect on G6PD.

In the guinea pig the evidence clearly points to 6PGD and not G6PD as the enzyme which shows earliest changes in response to increased requirements for corticosteroid production. It shows most marked increase after corticotrophin, is independent of adrenal growth and is sustained during the whole period of corticotrophin administration (Figs. 17 and 18).

Thus the principal NADP linked enzyme found to increase in the human adrenal cortex after corticotrophin administration is G6PD and in the rat and guinea pig adrenal it is 6PGD. But the main observation is that in all species studied one or more of the pentose phosphate cycle dehydrogenases doubles its activity after corticotrophin stimulation of the adrenal.

3. Relationship of the present findings to Haynes' Theory of Corticotrophin Action.

The principal features of Haynes' theory of corticotrophin action are summarised in Fig. 7. Its main point is that corticotrophin starts a chain of reactions which result in an accelerated breakdown

of glycogen to G6P in the cells of adrenal cortex. G6P is then assumed to enter the pentose phosphate cycle and this would result in an increase in production of $NADPH_2$, which is believed to be sufficient to cause increased corticosteroid production.

There are some weaknesses in this theory, such as a lack of sufficient glycogen in the adrenal to explain sustained action of corticotrophin (Noble and Papageorge, 1953). Also, while Haynes was able to show an increase in phosphorylase activity following corticotrophin administration, he failed to show any increase in G6PD activity (Haynes and Berthet, 1957). Thus there is nothing in his work to suggest that G6PD plays a role in the mechanism of corticotrophin action, and all Haynes' findings could be explained by a supposition that G6P is broken down by the glycolytic pathway and two carbon fragments enter the Krebs' cycle, thus providing $NADPH_2$ under the actions of IGD which is very active in the adrenal cortex. However, the experiments reported here give concrete support to Haynes' suggestion that the pentose phosphate cycle dehydrogenases play an important role in the supply of $NADPH_2$ for corticosteroid biosynthesis. Short exposures (30-90 minutes) of the adrenal to corticotrophin, in the present experiments, comparable in time to those of Haynes and Berthet (1957) showed no demonstrable increase in G6PD activity in the human adrenal cortex, but a definite increase in the activity of this enzyme was seen after 20 hours or more of corticotrophin action.

It would appear that corticotrophin does indeed alter the balance of metabolic pathways diverging from G6P in favour of the pentose phosphate cycle, but the fact that a considerable period of time must elapse for this effect to become evident makes it unlikely that the increase in G6P activity is the primary event in the mechanism of corticotrophin action. It is possible, however, that an early increase in G6PD activity is too small to be measured.

Viewed in the light of the ideas on adaptive enzyme formation, the increase in G6PD or 6PGD noted indicates that as a consequence of corticotrophin action on the adrenal cortex, a larger number of molecules of G6P enter the pentose phosphate pathway. This cannot be taken as final proof of Haynes' hypothesis, since other difficulties remain. Although Haynes and Berthet (1957) have shown an increase in phosphorylase activity following corticotrophin administration it cannot be assumed that G6P molecules thus produced will enter the pentose phosphate pathway. For instance, phosphohexose isomerase is very active in the adrenal cortex and its action will result in diverting G6P molecules into the glycolytic pathway instead of the pentose phosphate cycle. Similarly, if glucose-6-phosphatase is present in high concentration its action would deprive the increase in phosphorylase activity of most of its significance, and glycogen synthesising enzymes will also counteract the effect of phosphorylase. Korits and Foron's reservations concerning Haynes' theory have already

been mentioned on page 31. Nevertheless, this theory, when combined with that of Hechter and colleagues (page 30), seems to be the best available hypothesis concerning the mechanism of corticotrophin action. The results obtained in the experiments reported here give it further support.

4. Distribution of the Enzymes within the Cell.

The distribution of enzymes in subcellular fractions shows that the NADP linked dehydrogenase activity is present mainly in the soluble part of cytoplasm of the cells in the human adrenal cortex, as it is in other mammalian tissues. Glock and McLean (1955) found G6PD and 6PGD exclusively in the soluble fraction of rat liver cells. Butler and Lardy (1958) found ME only in the same fraction of pidgeon liver cells. Ernster and Navazio (1957) state that the major part of the NADP specific ICD present in liver cells is recoverable from the soluble fraction, and only 10% is located in the mitochondria. The findings reported here are very similar, and only ICD could be identified in the mitochondria. The only other study on the intracellular distribution of these enzymes in human tissues is that of Villee and colleagues (Villee et al., 1960), who found a similar distribution of G6PD and ICD in the placenta.

Schneider and Hogeboom (1956) comment on the mitochondrial membrane which may limit the access of substrates to mitochondrial enzymes. Thus several enzymes, e.g., acid phosphatase (Berthet et al., 1951)

and glutamic dehydrogenase (Hogeboom and Schneider, 1953), have been shown to manifest their activity only after this membrane has been destroyed. That this is not the reason for the inability to demonstrate G6PD, 6PGD and MB activity in the mitochondria is shown by the results obtained after the mitochondrial membrane has been destroyed by alternate freezing and thawing, or by subjecting the mitochondrial suspensions to hypotonic conditions. Only ICD showed an increase in activity after such procedures.

5. Distribution of G6PD in the zones of the Adrenal Cortex.

The distribution of G6PD throughout the human adrenal cortex found here shows a large measure of agreement with the observations of Greenberg and Glick (1960), that the increase in G6PD activity after corticotrophin administration occurs only in the fasciular-reticular border zone of the rat adrenal cortex. It should be noted, however, that the corticotrophin stimulation used by them was shorter (3 hours) and it is possible that a general increase in G6PD activity occurs later throughout the cortex. Histochemical demonstration of G6PD also shows that this enzyme is most abundant in the "mid fasciculata" of the rat adrenal cortex (Cohen, 1959; 1961), but this method cannot detect any change in enzyme activity after stress (Cohen, 1961).

The finding that G6PD is particularly active in the cells of zona fasciculata which became converted into compact cells, with the peak of activity at the interface between clear and compact cells,

helps to interpret the morphological changes occuring in the human adrenal cortex after corticotrophin administration. For instance, it gives chemical support to the histochemical observations of Symington et al. (1956) that the appearance of compact cells is not a degenerative phenomenon but on the contrary the compact cells are metabolically highly active. Secondly it pinpoints the compact cell-clear cell border as the site of the maximal activity; it is easy to imagine that as the cells become depleted of stores of lipid materials in response to increased demands for corticosteroid biosynthesis and growth, the wave of maximal activity advances towards the periphery from its normal position deep in the inner layers of the cortex. Another point illustrated is that in the normal unstimulated gland the activity of G6PD is uniformly distributed between the zones reticularis and fasciculata. It could be suggested therefore, that in an unstimulated gland both zones are synthesising lipids at a similar rate; in the zona reticularis steroids are secreted into the blood stream as soon as formed and no obtainable lipid complexes are seen in the cells. In the zona fasciculata, however, production of steroids exceeds output so the final stages of corticosteroid formation are not completed but the steroid is stored in the form of cholesterol derivatives. The histological appearance is the result of the balance between formation and "release" of cholesterol. "Release" in this context includes its conversion to steroid hormones, which

are never stored in the cell.

CONCLUSIONS

1. Glucose-6-phosphate dehydrogenase increases markedly in the human adrenal cortex after treatment of the patient with corticotrophin. This increase does not depend on the intensity of the stimulation of adrenal growth seen after corticotrophin administration. It is concluded that the increase in this enzyme activity is associated principally with the increase in corticosteroid hormone production.

2. The increase in glucose-6-phosphate dehydrogenase activity is most marked in those cells of the adrenal cortex which become depleted of stored lipids under the action of corticotrophin, with a peak of activity at the new clear cell - compact cell border.

3. Certain other NADP linked dehydrogenases of human adrenal cortex show no such increase after corticotrophin administration.

4. Those properties of those enzymes that were studied here were the same as the properties of the analogous enzymes described in other tissues. In particular, the intracellular distribution of these enzymes is exactly the same as in animal and other human tissues.

5. In rat and guinea pig adrenal gland glucose-6-phosphate dehydrogenase also increases after corticotrophin administration *in vivo* but the increase is less marked than that of 6-phosphogluconate dehydrogenase. It becomes more marked, however, when adrenal growth rate is more enhanced.

6. These findings are taken to supply some missing evidence for the correctness of Haynes' theory for the mechanism of corticotrophin action.

SECTION III

SECTION III

EFFECTS OF CORTICOTROPHIN ON THE HUMAN ADRENAL CORTEX IN VITRO

It is an interesting property of the adrenal cortex of many species that if placed under favourable conditions after removal from the body, it continues to secrete the same steroid hormones as it produces under normal conditions. Thus if the adrenal of a rat is cut into several small pieces immediately on removal from the animal and placed in a medium at the physiological pH, containing correct amounts of inorganic salts (Krebs-Ringer Medium) and glucose, and then shaken at 37°C in an atmosphere of oxygen and carbon dioxide, steroids will be found to be secreted into the medium. These steroids have the physical, chemical and biological properties of the principal adrenocortical hormones (Saffran et al., 1952; Elliott et al., 1954; Elliott and Schally, 1955). Similar observations have been made on adrenal tissue from pigs (Haines, 1952), ox (Haynes and Berthet, 1957), mouse (Hofmann, 1956), chicken (Rose, 1960), man (Griffiths, 1960) and some other mammalian species.

This is not merely release of preformed hormones, already present in the cell; it has been shown for several species that the amount of hormone present in the adrenal cell is less than the amount which appears in the medium on incubation for one hour (Holtzbauer, 1957; Elliott et al., 1954; Haynes and Berthet, 1957). A further point showing that we are not dealing with the simple process of "washing away" corticoids from the cell, a passive non-physiological process,

to the finding that addition of corticotrophin results in markedly increased steroid hormone production (Saffran et al., 1952). It seems likely, therefore, that the normal property of the adrenocortical cell of converting precursors (such as acetate or cholesterol esters) into steroid hormones by a process accelerated by corticotrophin is retained under the above conditions.

These findings offer us a valuable system for studying the survival and function of highly specialized cells under exactly defined and easily reproducible conditions. In particular we can attempt to learn something about the conditions necessary for steroid production by the adrenal cortex, about the nature of the hormones produced, the factors and conditions enhancing steroid output, those suppressing it, the characteristics of the response to corticotrophin, and the mechanism of this response.

The conditions necessary for steroid production have been well established for the adrenals of several animal species; in particular, for the ox and the rat. Saffran and his colleagues at McGill University (Saffran et al., 1952; Saffran and Bayliss, 1955; Saffran and Schally, 1955; Elliott et al., 1954; Elliott and Schally, 1955) give detailed descriptions of the properties of in vitro production of steroids by rat adrenals, and their finding that the steroid output is proportional to the logarithm of the dose of corticotrophin added (over a certain range only), prompted them to suggest the use

of rat adrenals *in vitro* for assaying the potency of corticotrophin.

Human adrenal cortex, however, has been studied much less thoroughly; the studies reported in the literature were concerned principally with the output of steroids by adrenals showing some pathological feature such as Cushing's syndrome, Conn's syndrome, etc. (Dyrenfurth *et al.*, 1960; Bailey *et al.*, 1960) and often attempted to correlate the pattern of steroids produced *in vitro* with the clinical picture of the patient - on the whole with little success, although Venning and her colleagues (1962) claim that *in vitro* secretion of cortisol is greater by adrenal tissue from cases of Cushing's Syndrome (5 cases) than by adrenal tissue from cases of breast carcinoma (1 case, with single reading). It has emerged from these studies, however, that the steroids with "glucocorticoid", salt retaining and androgenic properties which can be isolated from adrenal glands and are found in adrenal venous blood, can also be demonstrated in the medium after incubation of human adrenal cortex. These steroids were found in varying proportions, but the principal steroid produced by the human gland is cortisol, with lesser amounts of corticosterone. Aldosterone, cortisone and 11β-hydroxy-androstenedione were also found by some workers who incubated relatively large amounts of adrenal tissue. Addition of corticotrophin to the incubation media increased the output of all these steroids.

Most workers did not concern themselves with the characteristics

of this response to corticotrophin, nor with the type of adrenocortical cell which produces the steroids. However, in a recent study Griffiths (1960) pointed out that the different zones of human adrenal cortex differ in their response to corticotrophin. He found roughly equal steroid output by incubated zona reticularis and zona fasciculata; however, the addition of corticotrophin resulted in a very marked increase in steroid output by the zona fasciculata, while the increase in steroid output by the zona reticularis was very slight. The zona glomerulosa of the human adrenal cortex is too narrow and irregular to be isolated for these studies, although in animals this can be done with reasonable success, and zona glomerulosa has been shown to be the only site of formation of aldosterone in the ox (Ayres et al., 1956) and the rat adrenals (Giroud et al., 1956). On the basis of his experiments on the human adrenal cortex, Griffiths has reached the conclusion that the fasciculata clear cell zone is particularly sensitive to corticotrophin. It is probably therefore the region of the gland which responds to sudden demands on the adrenal cortex, as in stress or after corticotrophin administration to the patient.

Experiments reported in Section II furnish yet another example of the importance of the reduced co-enzyme, $NADPH_2$, in the action of corticotrophin on the adrenal cortex. Since it has proved impossible to separate completely the growth promoting activity from steroidogenic activity of corticotrophin under *in vivo* conditions, the problem has

been approached under *in vitro* conditions, since here the steroidogenic effect is not accompanied by tissue growth. Monfold *et al.* (1959) found that enzymic reduction of NADP is markedly inhibited by 2-adenylic acid (2-AMP). This nucleotide and its analogues were therefore added to incubated slices of human adrenal cortex in order to test whether they have a suppressing effect on corticoid production. Another adenine nucleotide, adenosine-3',5'-cyclic monophosphate (3',5'-AMP), has been shown to have a steroidogenic effect on sectioned rat adrenal *in vitro* similar to that of corticotrophin (Haynes *et al.*, 1959). The effect of this compound was therefore studied on slices of human adrenal cortex, and corticotrophin like activity of insulin and vasopressin were also looked for. Insulin has been shown to increase intracellular concentration of glucose (Narahara *et al.*, 1960) followed by its phosphorylation to G6P, and on the basis of Haynes' theory of corticotrophin action (Fig. 7) one would expect that this should result in increased corticoid production. There are no theoretical reasons for suggesting that pitressin has steroidogenic properties. This has been claimed to be the case, however, under *in vivo* conditions. Royce and Sayers (1958) found that pitressin caused depletion of ascorbic acid in hypophysectomised rats, and Hilton *et al.* (1959) perfused directly adrenal glands in dogs and found sixfold increase in cortisol secretion, which they believe is independent of the increase in blood pressure produced by vasopressin.

In order to pursue the above aims it was thought necessary to study the properties of corticoid production by slices of human adrenal cortex and to investigate the characteristics of the response to corticotrophin *in vitro*. Since the principal effect of corticotrophin is on the clear cells of the zona fasciculata, the experiments to be reported refer to such tissue, unless otherwise stated.

Opportunity was also taken to study corticoid production *in vitro* by slices of human adrenal glands which showed structural (adenomata) or functional derangements (Cushing's Syndrome).

MATERIALS AND METHODS

Tissue Preparations.

The human adrenal glands studied in this section were obtained from patients undergoing bilateral adrenalectomy for advanced breast carcinoma, with the exception of two glands obtained from patients with Cushing's Syndrome. In some cases the patient received 40 I.U. of corticotrophin twice daily for four days immediately before the operation and these cases are indicated in the text.

Each gland was placed on ice immediately on removal and taken to the laboratory where it was carefully dissected free from adherent fat. The two opposed layers of the cortex were separated from each other by gentle traction, also removing and discarding the medulla at this stage, and then divided into small rectangular pieces approximately 1.5 cm. x 0.7 cm. The pieces were sliced in a modified Studio-Riggs microtome, giving two slices from each piece, each approximately 0.5 mm. thick. The inner slices, of brown colour, were pooled as "reticularis", the outer slices were yellow and are referred to as "fasciculata". Microscopical examination of haematoxylin and eosin stained sections confirmed that naked-eye separation on the basis of colour corresponds exactly to the histological type of cell.

In some experiments guinea pig adrenals were used. In these cases the animals were killed by a blow on the head and the adrenals were removed immediately and dealt with in the same way as the

human glands. Medulla could not be separated from the cortex, however, and the adrenals were cut into small pieces with scissors instead of slicing.

Incubation of slices.

The procedure for incubation of tissue slices and extraction of medium was based on the method of Saffran and Payllen (1953).

Approximately 50 mg. lots of each type of slice (reticularis or fasciculata) were accurately weighed on a torsion balance and placed in small bottles containing 1.5 ml. of Krebs Ringer Bicarbonate medium at pH 7.4. The medium contained 200 mg.% of glucose. The bottles were placed under a jet of 95% O_2-5% CO_2 mixture for 5 minutes, and each bottle was closed with a tightly fitting plastic cap. The aim was to have four bottles for each reading, but at times it was only possible to have triplicates.

The containers were placed in a water bath at 37°C and shaken for one hour. At the end of this time the medium was withdrawn and discarded, and replaced by another 1.5 ml. of lot of fresh medium, oxygenated, and incubated again, this time for two hours.

The first incubation is referred to as "preincubation" and is necessary for the following reasons:

(a) It allows the adrenal tissue to recover from the trauma sustained in the removal and slicing of the adrenal and perhaps from residual effects of endogenous corticotrophin (Saffran et al., 1952).

(b) Miscellaneous lipids which interfere with steroid estimations are passively washed out from cells damaged in slicing.

The medium withdrawn at the end of the subsequent two hours was kept and analysed for steroid output by each batch of slices. This may be referred to as "basal" steroid output, that is, the output when the slices are not stimulated by added ACTH.

The slices were then incubated for a third period (2 hours). On this occasion corticotrophin and / or some other substance were added to the medium. The medium was analysed for steroids.

The purpose of incubating each batch of tissue slices for the first two hour period without any additions was to obtain a baseline for further readings. The effect of additions could then be obtained from the difference in output with and without the substance added.

On some occasions the slices were incubated for several further two hour periods. At the end of the final incubation the slices were placed in 10% neutral formalin for fixation and processed for histology by standard methods of paraffin embedding and haematoxylin and eosin staining.

Extraction and measurement of steroids.

Steroids were extracted from the incubation media by methylene chloride, evaporated to dryness under a stream of nitrogen and redissolved in 0.4 ml. of pure ethanol. The optical density of the solution was read at 229, 240 and 255 mμ in a Unicam S.P. 500 spectrophotometer

with a microcell attachment using 0.3 ml. silica cells. The amount of steroid present was calculated from the formula:

$$\text{Optical Density (O.D.) at 240 m}\mu = \frac{\text{O.D. at 225 m}\mu + \text{O.D. at 255 m}\mu}{2}$$

(Allen, 1950) and compared with the value similarly obtained for a known amount of cortisol. Extraction controls and blank extractions were also routinely performed. Blanks (i.e., extraction of medium in which adrenals were not incubated) were always close to zero; extraction controls (a known amount of cortisol dissolved in the medium and then extracted as above) gave approximately 90% recovery.

The alcoholic solutions were retained after the U.V. reading. The aliquots of each group of readings were pooled, evaporated again, and redissolved in 0.02 ml. of ethanol, then applied to paper for chromatography. Known amounts of cortisol and cortisosterone were also applied as standards for identification. The chromatograms were run in a Bush-Sandberg system where the mobile phase is benzene, and the stationary phase methanol-water (Bush, 1952). After two hours the chromatograms were dried, and U.V. absorbing spots were visualised under an ultraviolet light producing lamp. The chromatograms were then sprayed with an alkaline solution of blue tetrazolium to visualise reducing substances (Elliott et al., 1954).

The mobility in methanol-benzene in relation to standards, U.V. light absorption (representing the unsaturated-α,β-ketone group of the steroid nucleus) and the reducing property of the α-ketol side

chosen were taken as pointers to the identity of steroids produced by the adrenals (Elliott and Donally, 1955).

In some experiments the spots noted in U.V. light were eluted with methanol. The amounts of steroid were determined quantitatively after development of colour with blue tetrazolium, using the S.P. 500 spectrophotometer at 510 mμ.

Enzyme Assay.

Glucose-6-phosphate dehydrogenase was assayed by the method of Glock and Nelson (1953) with modifications noted on page 67.

Materials.

Corticotrophin used in the *in vitro* experiments was a lyophilised preparation of Corticotrophin A (Organon) with potency of approximately 100 I.U./mg. For *in vivo* administration current commercial preparations of long acting Armour corticotrophin were used.

Adenosine-3',5'-cyclic phosphate (3',5'-AMP), adenosine 2'-monophosphate (2'-AMP), and adenosine 5'-monophosphate (5'-AMP) were obtained from Schwarz Bio Research Inc., (U.S.A.).

Insulin was a Burroughs Wellcome and Company (London) product.

Vasopressin was obtained from Parke, Davis and Company, (London). This is a posterior pituitary extract.

RESULTS

1. Secretion of steroids by slices of human adrenal cortex.

Slices of human adrenal cortex were found to secrete cortico-steroids for at least nine hours of incubation, and were able to respond to corticotrophin during the whole of this time (Fig. 20). The steroid output by each slice remains constant within that time, but increases when corticotrophin is added. In the absence of corticotrophin, the zona reticularis and the zona fasciculata secrete approximately equal amounts of corticosteroid, that is, about 10 µg. total U.V. light absorbing steroids by 100mg. of adrenal tissue in two hours (c.f. Fig. 38). The observation of Griffiths (1960) that the zona reticularis of human adrenal cortex responds little if at all to corticotrophin while the clear cells of zona fasciculata show marked increase in steroid output has been confirmed in eight separate experiments using at least three incubations for each reading (c.g., see Fig. 38).

The secretion of corticosteroids *in vitro* is completely inhibited by addition of cyanide at 10^{-3}M concentration (Fig. 21).

Slices cut from glands obtained at post-mortem do not secrete steroids into the medium, nor can they be stimulated to do so by corticotrophin (Fig. 21).

The method of measurement employed in these experiments estimates lipids with an absorption peak at 240 mµ. Tests performed on the

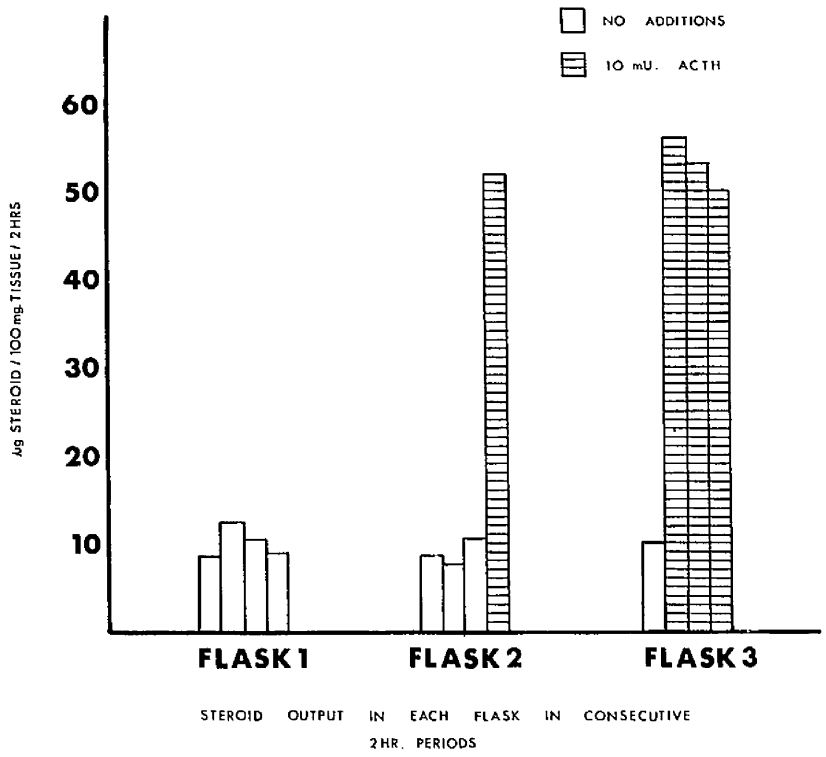


Fig. 20. Output of U.V. absorbing lipids by slices of the fasciculata zone of the human adrenal cortex. The output in the first hour was not measured.

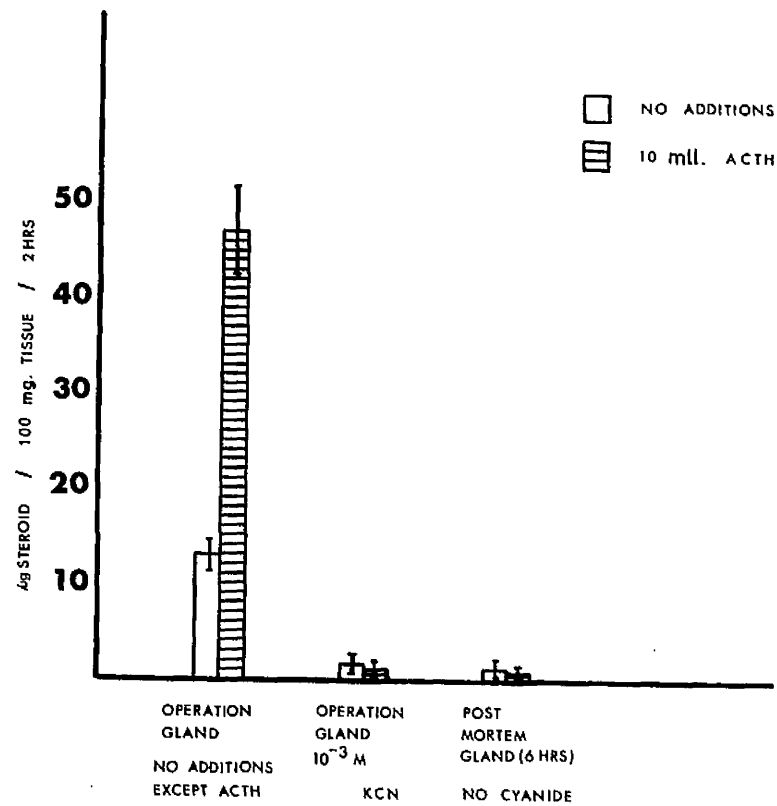


Fig. 21. Output of U.V. absorbing lipids by slices of the fasciculata zone of the human adrenal cortex. Effect of cyanide and lack of output by slices from a gland obtained post-mortem.

lines of Saffran and colleagues (1952) and more recently of Griffiths (1960) demonstrated that these lipids have a reducing ketol side chain (blue tetrazolium reaction) and chromatographic properties of cortisol and corticosterone (Fig. 31). The principal steroid found on paper chromatography in benzene - methanol-water was cortisol with approximately 1/3 that amount of corticosterone. Faint traces of cortisone were detected on some chromatograms but if other steroids were present, the amount released by 200 mg. of adrenal tissue in two hours was not sufficient for detection in the chromatographic system used.

In spite of the continuous production of steroids and the ability to respond to corticotrophin during the incubation time it is still possible that cell survival in the slices is not complete. Some cells could be undergoing irreversible changes while still retaining the property of steroid production. This was investigated in two ways. Firstly, the enzyme shown in Section II to be related to steroid production by the adrenal cortex, glucose-6-phosphate dehydrogenase, was measured before and after incubation; it was found that after six hours of incubation the enzyme activity was only one third of the original value. Secondly, the slices were examined histologically before (Figs. 22 and 24) and after the incubation (Figs. 23 and 25). It is evident that many cells are disrupted and advanced nuclear changes indicative of cell death are a very frequent occurrence

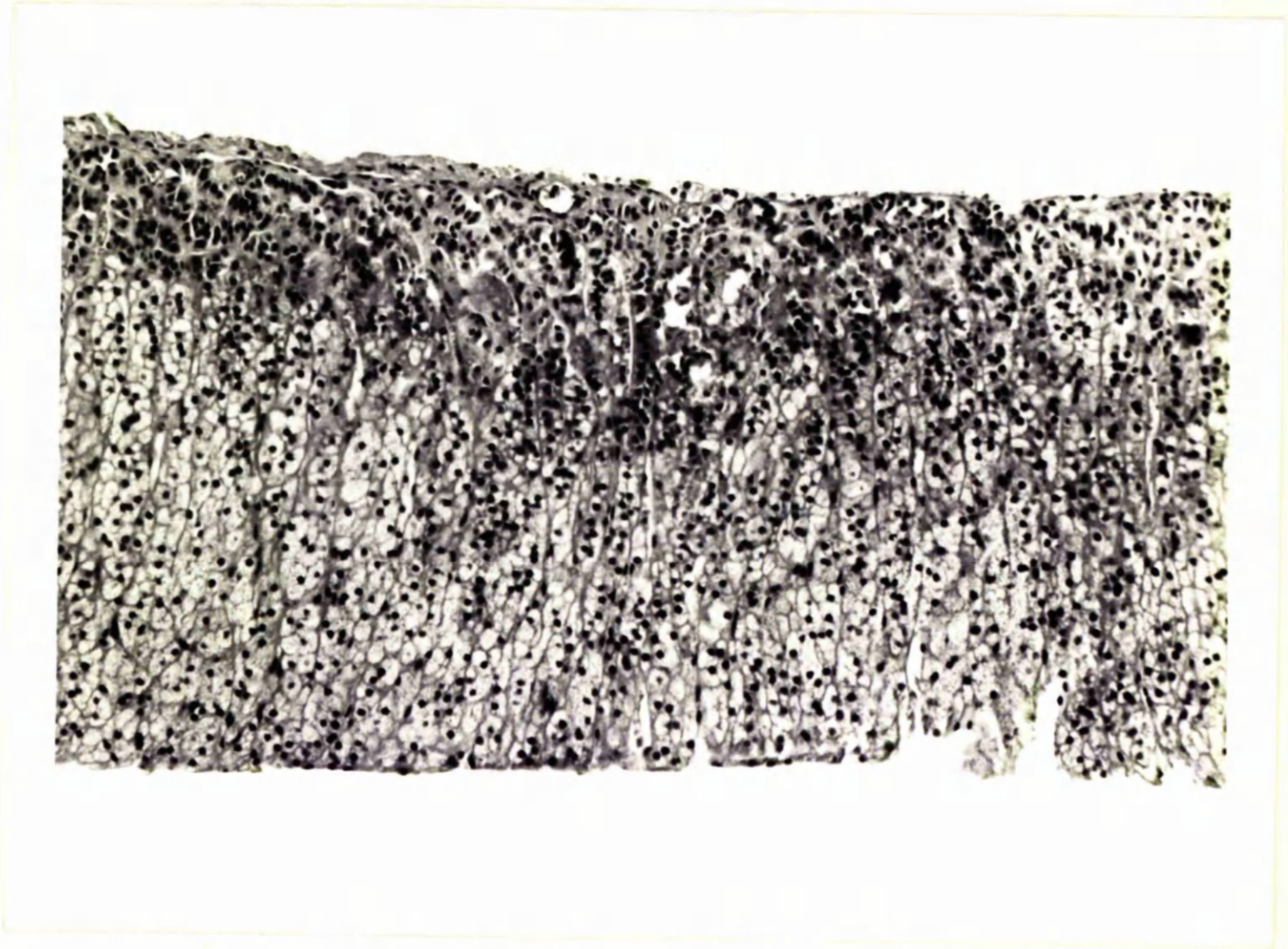


Fig. 22. A slice of zona fasciculata of human adrenal cortex before incubation. Stained by H. and E. x 175.

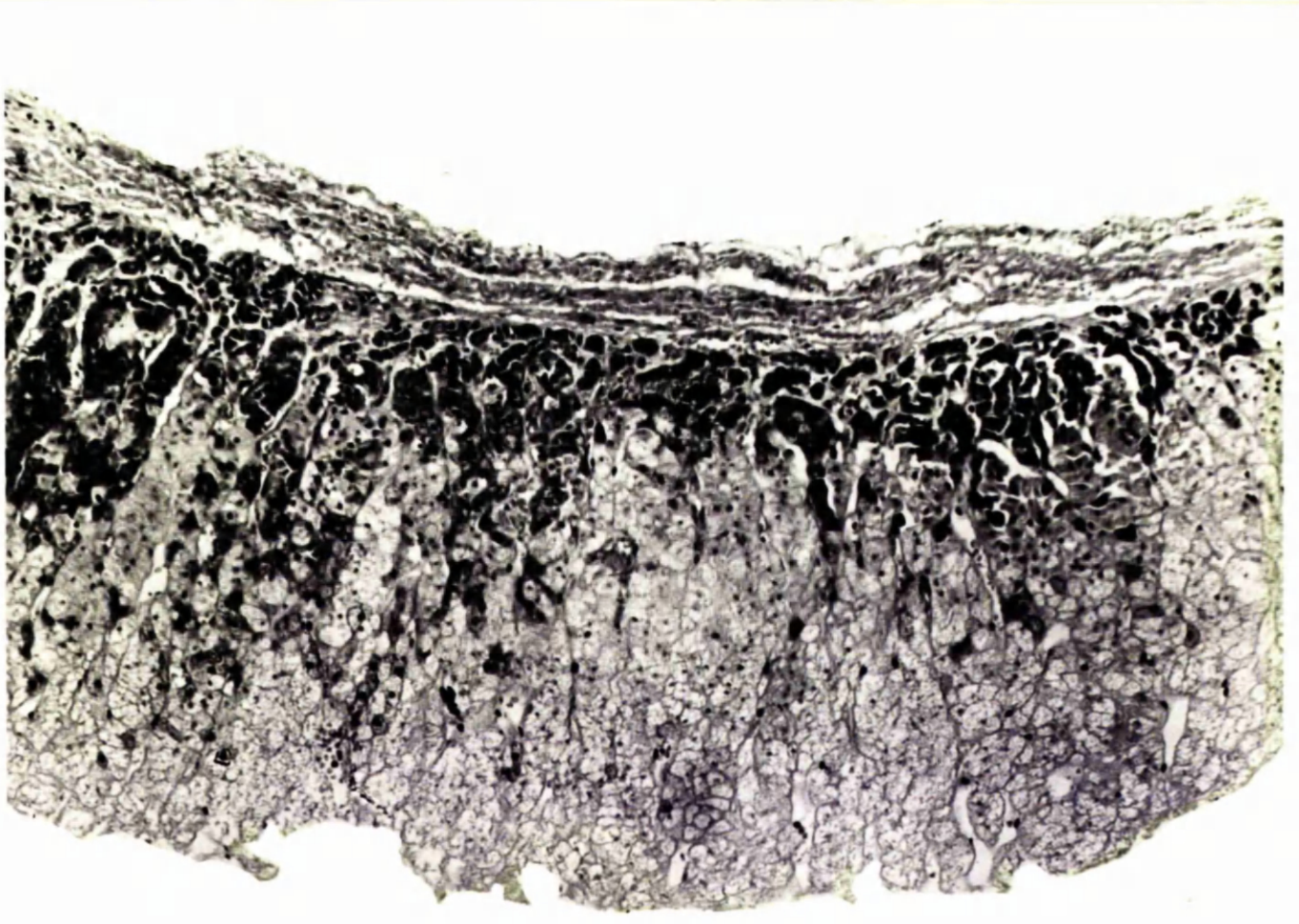


Fig. 23. A slice of zona fasciculata similar to that shown in Fig. 22 but after incubation for 5 hours. The clear cells are disrupted but zona glomerulosa cells survive structurally, as do some compact cells in the zona fasciculata. H. and E. staining x 150.

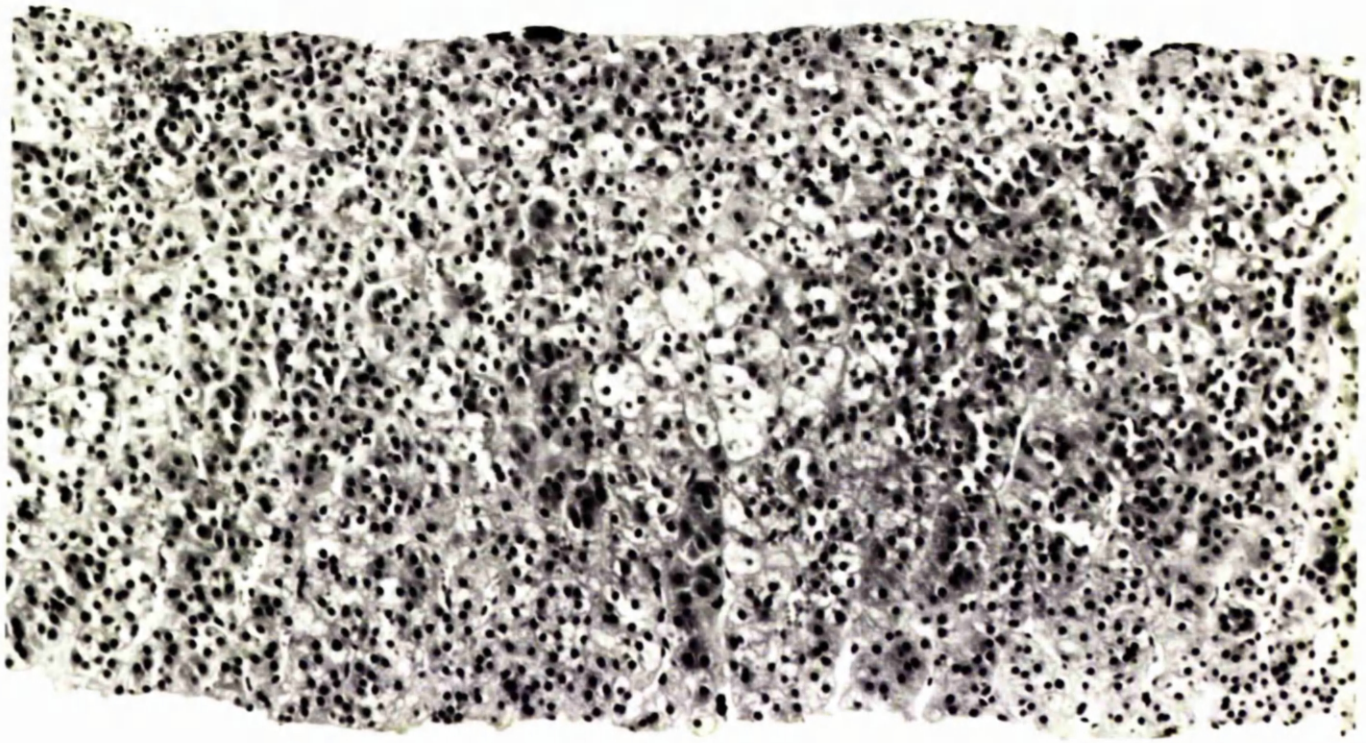


Fig. 24. A slice from the inner layer of human adrenal cortex, mainly zona reticularis, before incubation. H. and E. staining x 175.

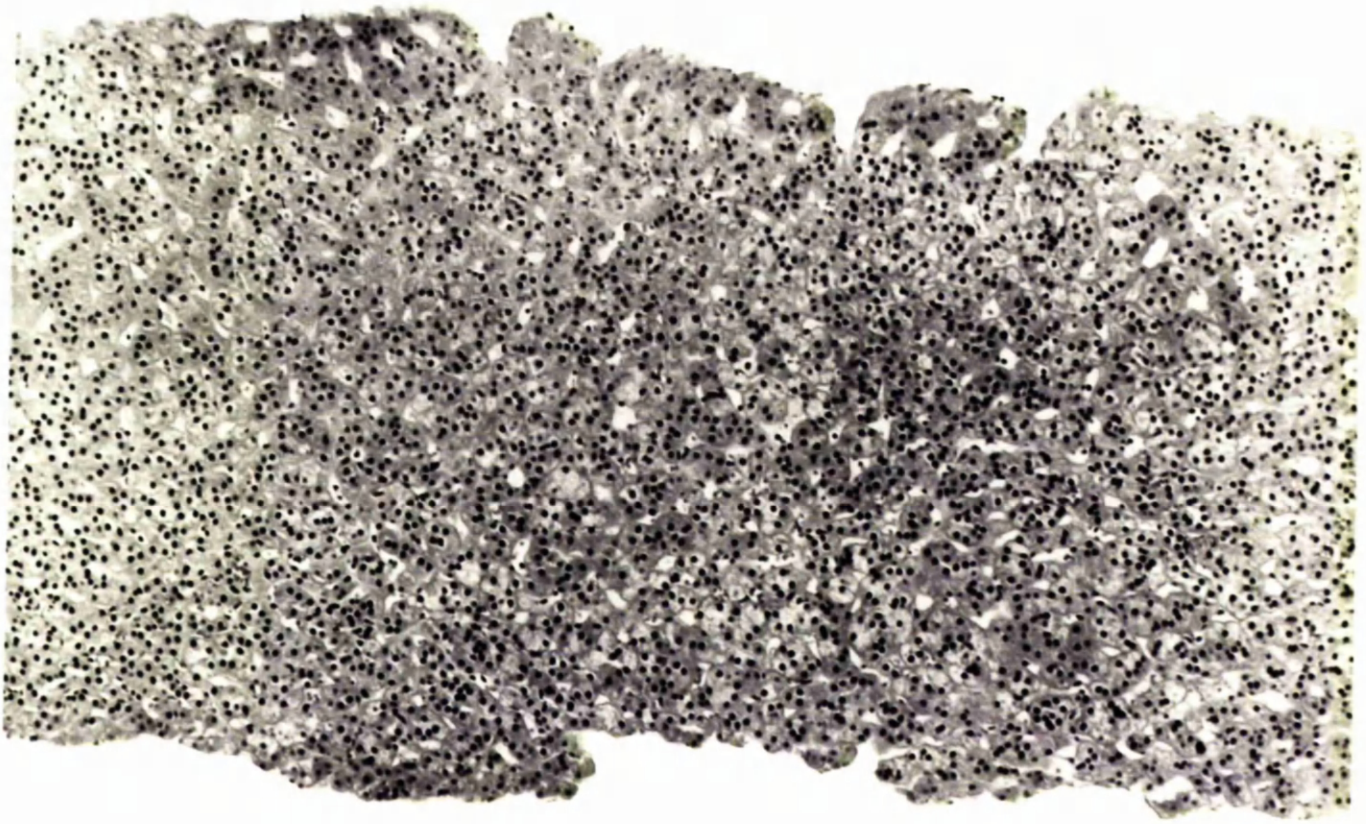


Fig. 25. A slice similar to that shown in Fig. 24 but after incubation for 5 hours. There is little cell disruption in this case. H. and E. staining x 125.

among the fat laden "clear cells" typical of the zona fasciculata (Fig. 24). This change was seen in the clear cells irrespective of whether corticotrophin was added to the incubation system or not. The eosinophilic "compact" cells of the zona reticularis remain intact (Fig. 25). The reason for this difference is not known.

2. Characteristics of the steroid output in response to corticotrophin.

It may be seen from Fig. 26 that a response can be detected when as little as 1×10^{-5} International unit of corticotrophin (or 0.01 mU) is added to 1.5 ml. of medium containing slices of human adrenal cortex. The maximal response is obtained with 0.1 mU/1.5 ml. medium.

As described for rat adrenals (Saffran and Schally, 1955) the response is linear when plotted against the logarithm of the dose of corticotrophin added. However, the limits are approximately 0.005-0.1 mU/1.5 ml. of medium, while the effective dosage is much higher for the rat (10-200 mU/1.5 ml.).

By contrast, guinea pig adrenals *in vitro* were found to require 10 units (i.e., 10000 mU) of ACTH for a detectable response.

Another characteristic of the response of the rat adrenal to corticotrophin (e.g. Ward, 1961) which has been found to apply to the human adrenocortical cell is the persistence of the stimulatory effect of corticotrophin after all traces of corticotrophin have been removed from the incubation medium. Fig. 27 shows the results of an experiment illustrating this point. It shows that the stimulation

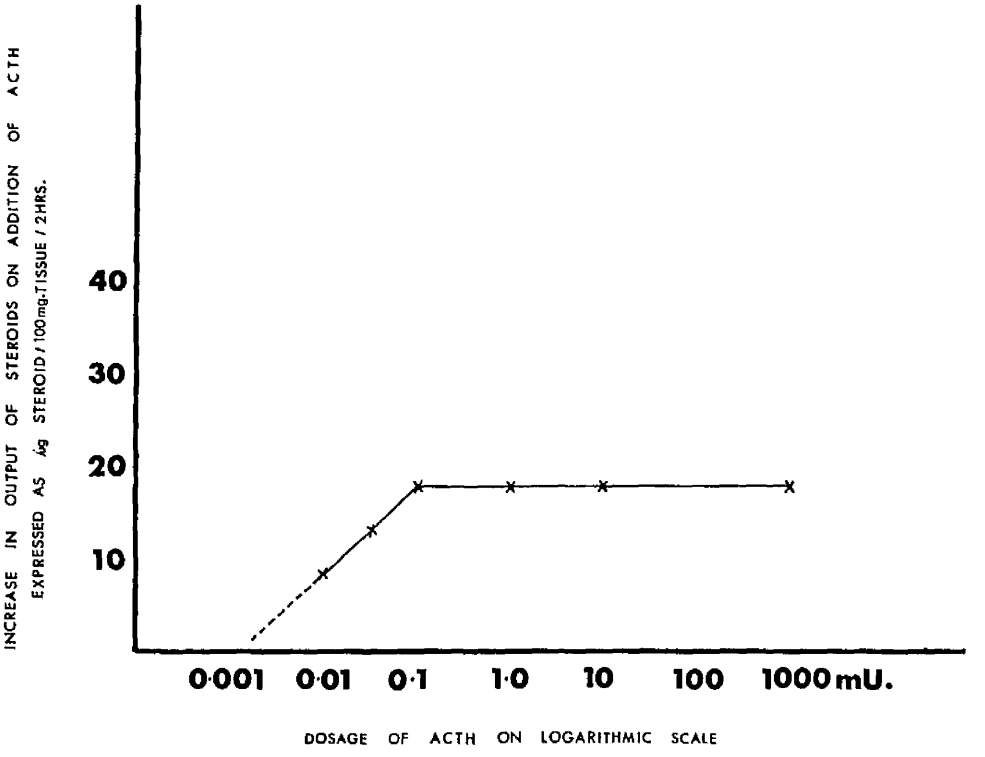


Fig. 26. Log dose - response graph for sensitivity of human adrenal cortex cells to corticotrophin.

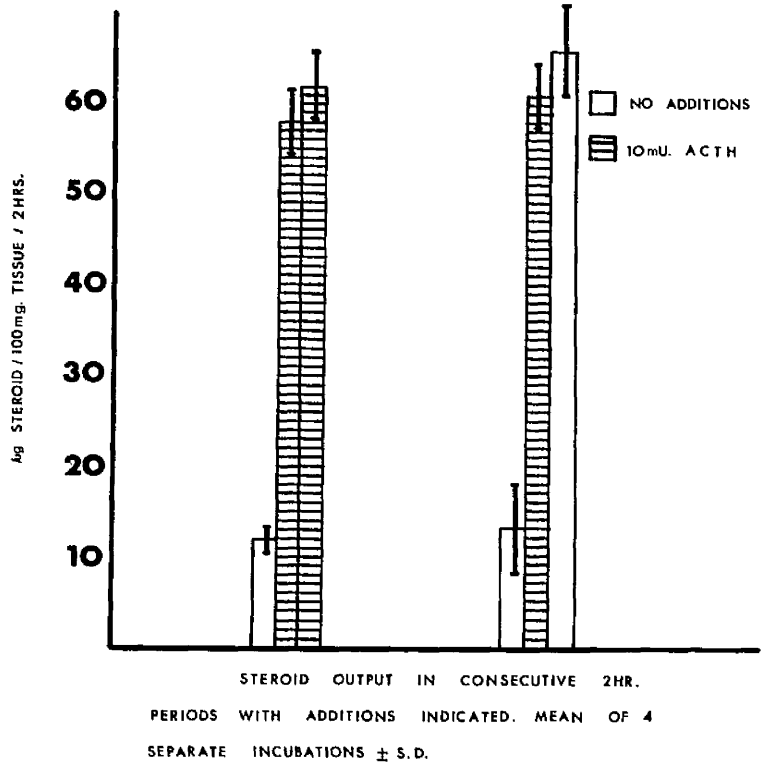


Fig. 27. Persistence of the stimulatory effect of corticotrophin on the cells of zona fasciculata of the human adrenal cortex.

caused by corticotrophin persists into the third two hour period of incubation, even though the medium has been changed and the adrenal slices washed thoroughly between the incubations, and is therefore unlikely to have been caused by traces of corticotrophin carried over on the slices.

The adrenal glands can be stored for at least twelve hours at 0^{o}C and when subsequently sliced the tissue will secrete steroids *in vitro* and respond to corticotrophin. Freezing the glands, however, to -20^{o}C for even five minutes results in a complete loss of the ability to respond to corticotrophin stimulation, although the output without corticotrophin is increased. This resembles the behaviour of rat adrenal tissue, as found by Koritz and Peron (1958).

3. Effects of adenine nucleotides, insulin and nitrogen on the secretion of steroids by slices of human adrenal cortex.

A. Inhibition of steroid output by adenosine 2'-monophosphate (2-AMP)

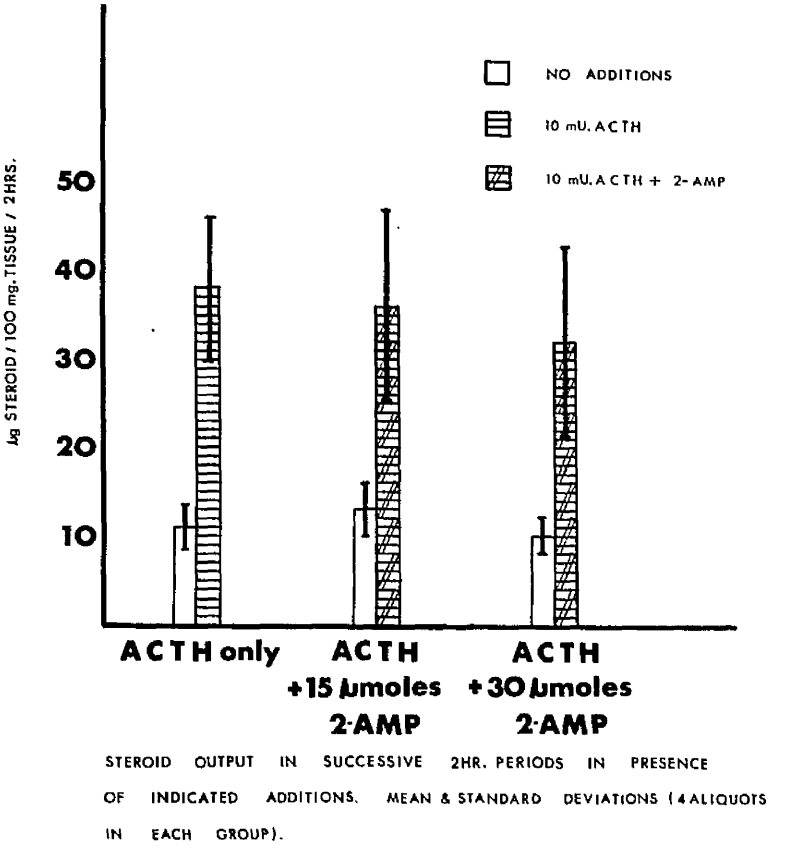
When 2-AMP is added to an adrenal cortex homogenate, $NADPH_2$ production is greatly diminished. If the concentration of 2-AMP is 30 µmoles/1.9 ml. the reduction of NADP in a homogenate proceeds at only 30% of its normal rate (Table 26). However, it may be seen from Fig. 28 that a similar concentration of this nucleotide in the incubation medium has not had a similar effect on corticosteroid production by adrenal slices.

The slight depression noted when 30 µmoles of 2-AMP were added

TABLE XXVI

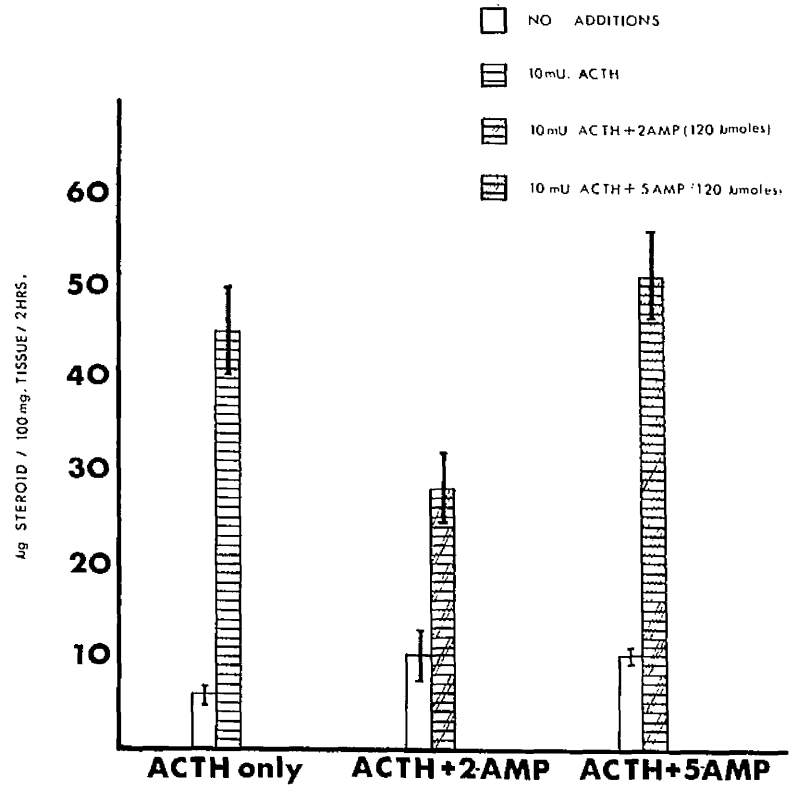
Inhibition by 2-AMP of NADP reduction in homogenates

Concentration of 2-AMP in µmoles per 1.5 ml.	%of maximum rate of NADP reduction
0	100%
4	82%
7.5	65%
15	41%
30	29%
60	15%



STEROID OUTPUT IN SUCCESSIVE 2HR. PERIODS IN PRESENCE OF INDICATED ADDITIONS. MEAN & STANDARD DEVIATIONS (4 ALIQUOTS IN EACH GROUP).

Fig. 28. Secretion of steroids in response to corticotrophin by slices of the zona fasciculata of the human adrenal cortex in the presence of adenosine-2'-monophosphate. There is no significant effect at these concentrations of the nucleotide.



STEROID OUTPUT IN SUCCESSIVE 2HR. PERIODS IN PRESENCE OF INDICATED ADDITIONS. MEAN OF 4 FLASKS ± S.D.

Fig. 29. Secretion of steroids in response to corticotrophin by slices of the zona fasciculata of the human adrenal cortex in the presence of high concentrations of adenosine-2'-monophosphate and adenosine-5'-monophosphate. At this concentration 2-AMP diminishes significantly the stimulatory effect of corticotrophin.

(Fig. 28) is not statistically significant. The concentration of 2-AMP was therefore increased to 120 µmoles per 1.5 ml. of incubating medium. There is a possibility that such a high concentration of 2-AMP could produce an effect on steroid output related not to NADP reducing enzymes, but merely to the toxic effects of the high concentration of the substance itself. To eliminate this possibility the same amount of adenylic acid (5-AMP) was added to another set of flasks; 5-AMP is chemically similar to 2-AMP, but has no specific effect on NADP reducing enzymes. The results are shown in Fig. 29. This time there occurred a significant decrease in the output of U.V. absorbing lipids when 2-AMP was added, but not when 5-AMP was substituted. The output in the presence of 2-AMP was reduced to approximately 60% of the original level and this decrease was statistically significant. In homogenates, however, similar degree of inhibition of NADP reduction can be achieved when only approximately 8 µmoles of 2-AMP are present (Table XXVI).

D. ACTH-like effect of 3',5'-AMP on human adrenal slices.

Fig. 30 shows that addition of 3',5'-AMP to slices of human adrenal cortex *in vitro* has the same effect as maximal stimulation with corticotrophin. It can be also seen that the effects of corticotrophin and 3',5'-AMP are not additive in human tissue: addition of these two substances together does not lead to a greater output than with either substance alone.

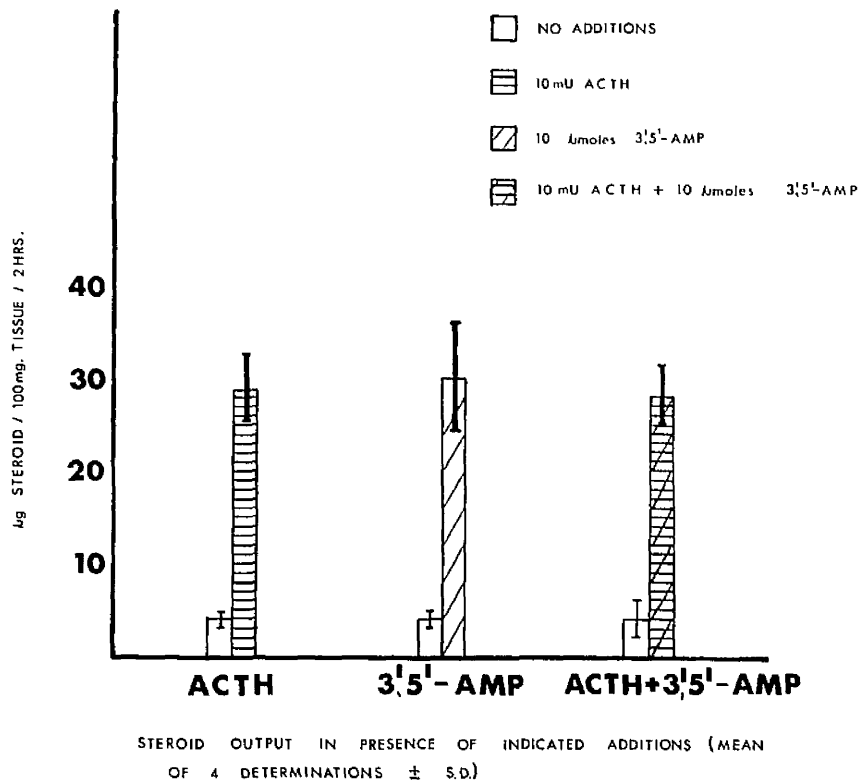


Fig. 30. ACTH-like effect of adenosine-3',5'-monophosphate on slices of the zona fasciculata of the human adrenal cortex.

Similar results were obtained in further four experiments.

The ultraviolet absorption measurements on methylene chloride extracts which gave the readings shown in the various figures were routinely checked by paper chromatography of these extracts to confirm the presence of cortisol and corticosterone in the extracts. The paper chromatogram for the experiment illustrated in Fig. 30 has been photographed and is shown in Fig. 31. It may be seen that not only the total amounts of corticosteroids secreted under the influence of ACTH and 3',5'-AMP are the same, but the principal steroids are in each case cortisol and corticosterone, with their ratios similar in each instance. This shows that 3',5'-AMP has exactly the same effect on human adrenal cortex as has corticotrophin in respect of the properties studied.

C. Effect of insulin on corticosteroid production.

Fig. 32 shows that insulin does not cause significant increase in steroid production and this finding was confirmed in further two experiments.

D. Effect of vasopressin on human adrenal cortex.

It is clear from Fig. 32 that vasopressin has no effect on the production of steroids by the human adrenal cortex in vitro.

4. Incubation of slices of adrenal glands showing special features.

Adrenal glands from two cases of Cushing's Syndrome became available for study while this investigation was in progress. The

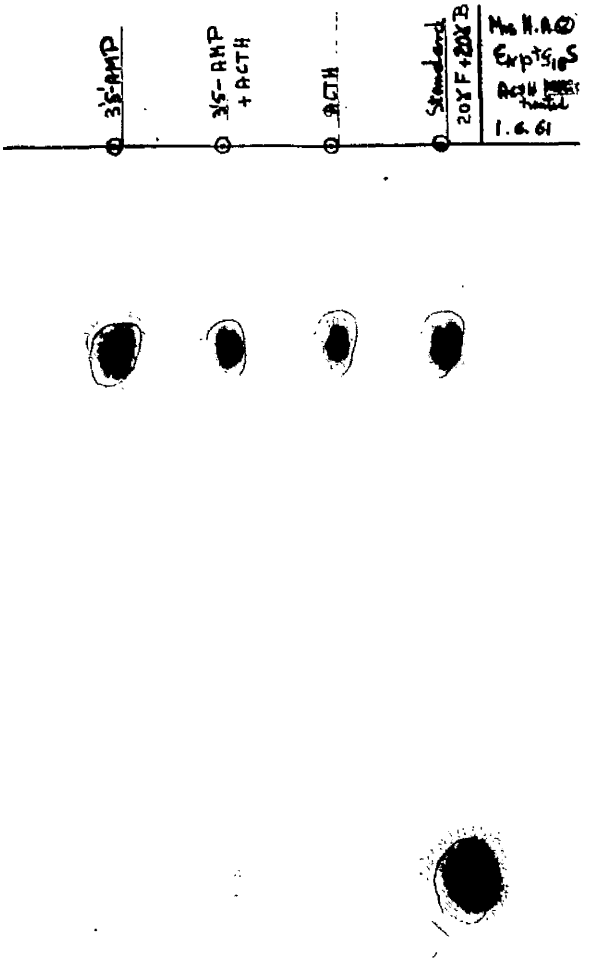


Fig. 31. A photograph of a paper chromatogram run on residues of the extracts the O.D. of which are shown in Fig. 30.

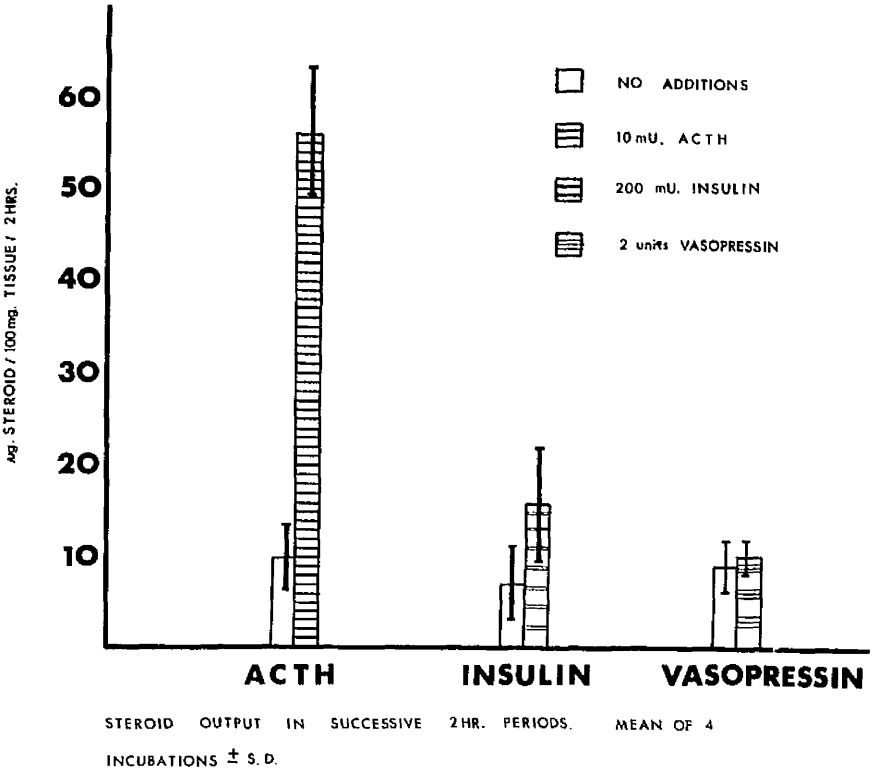


Fig. 32. Effect of insulin and vasopressin (pitressin) on steroid output by slices of the zona fasciculata of the human adrenal cortex.

in vitro steroid output by these glands was studied, and also by slices cut from adrenal adenomata from patients who had no endocrine symptoms. These results were compared with steroid output *in vitro* by adrenal glands obtained from patients to whom large doses of ACTH have been administered before adrenalectomy.

A. Output of corticosteroids by adrenal glands from cases of Cushing's Syndrome.

Fig. 33 shows that in the absence of corticotrophin the output of steroids by slices of adrenal glands from cases of Cushing's Syndrome is approximately the same as that of normal glands. When corticotrophin is added, the increase is definitely less marked than that obtained with slices from normal glands. Histological examination of these glands showed that the adrenal cortex from case 1 had broad zona reticularis (Fig. 34) while case 2 differs little from the appearances found in the normal glands (Fig. 35).

Paper chromatography of steroid extracts was performed on only one of these glands. The result was unusual in that the proportion of corticosterone was much higher than that generally produced by normal adrenal cortex, i.e., in this case concentration of corticosterone was equal to that of cortisol.

B. Secretion of corticosteroids by slices of adrenocortical adenomata

Six adrenal glands bearing cortical nodules were obtained from patients who showed no signs of adrenal hyperfunction. These

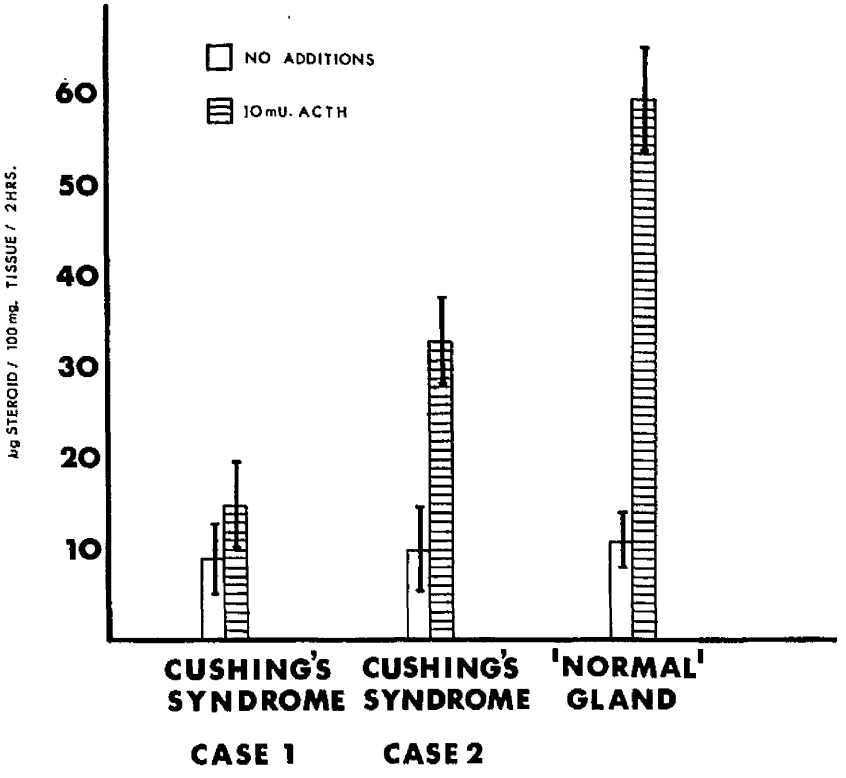


Fig. 33. Output of steroids by slices of the outer half of the adrenal cortex from two cases of Cushing's Syndrome compared with the output by a slice of the same region of a typical normal adrenal.

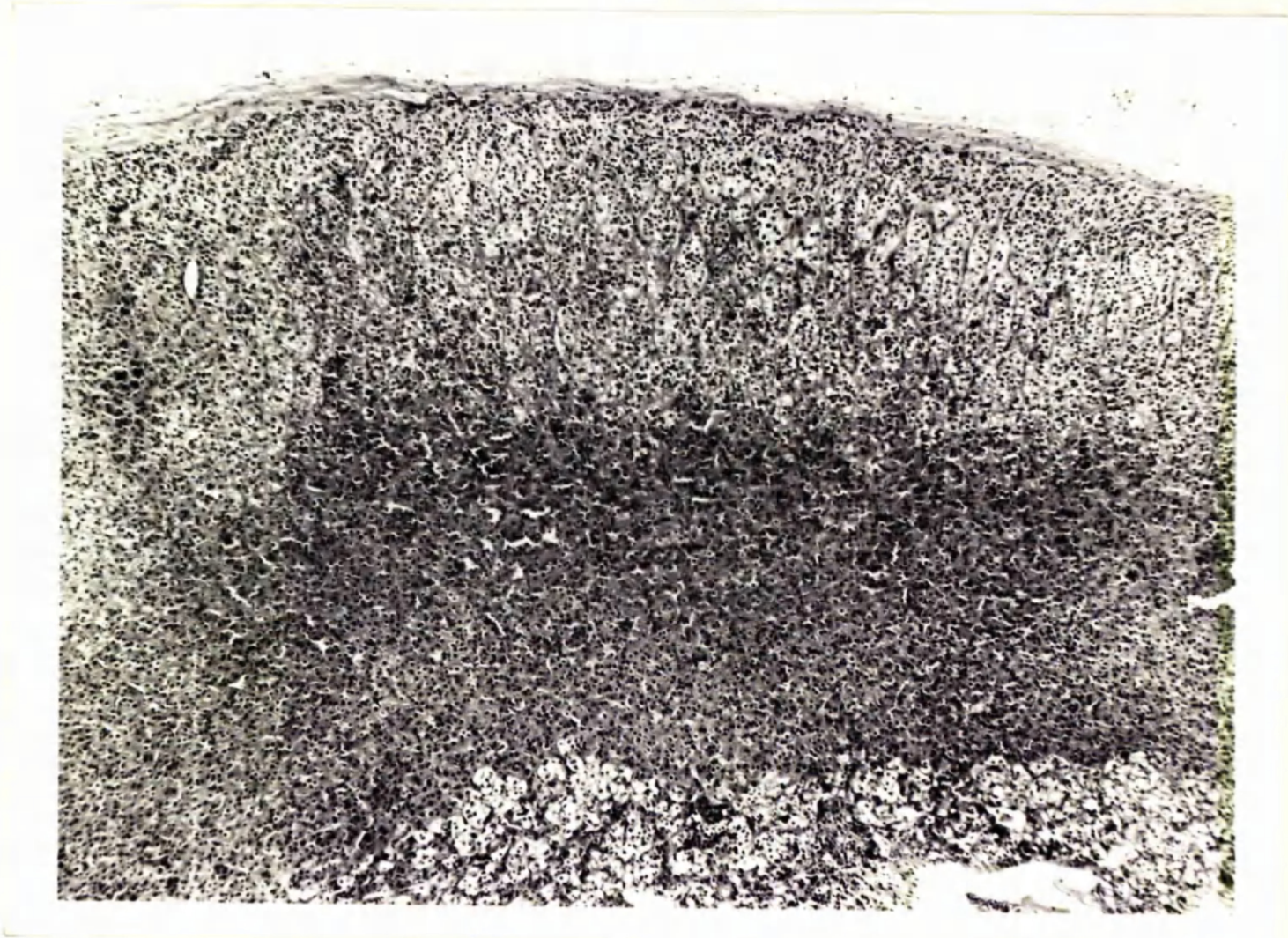


Fig. 34. Histological appearance of the adrenal cortex from Case 1 of Cushing's Syndrome. Compact cells account for more than a half of the thickness of the cortex. Stained by H. and E. x 75.

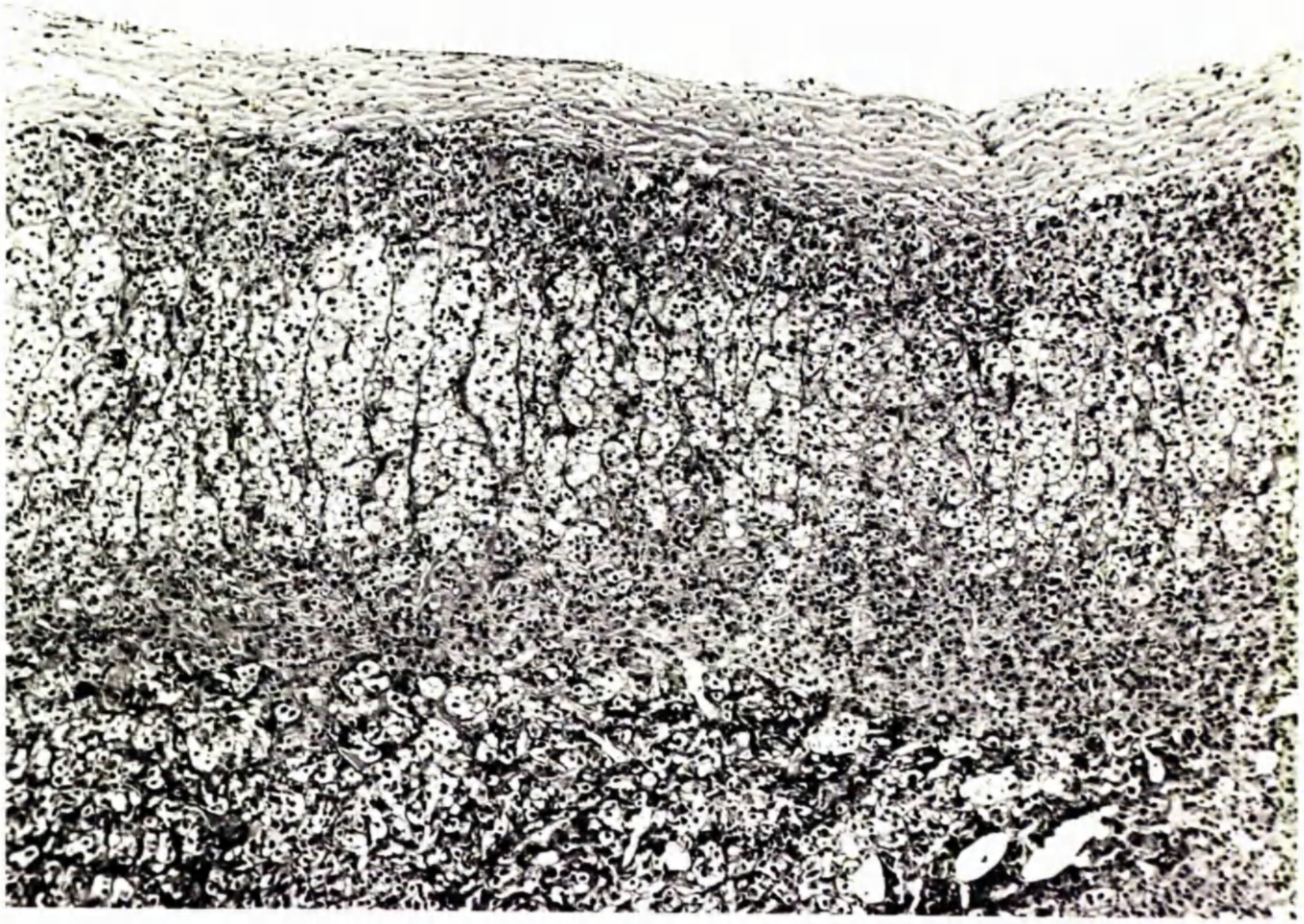


Fig. 35. Histological appearance of the adrenal cortex from Case 2 of Cushing's Syndrome. There is little difference from a normal gland. Stained by H. and E. x 90.

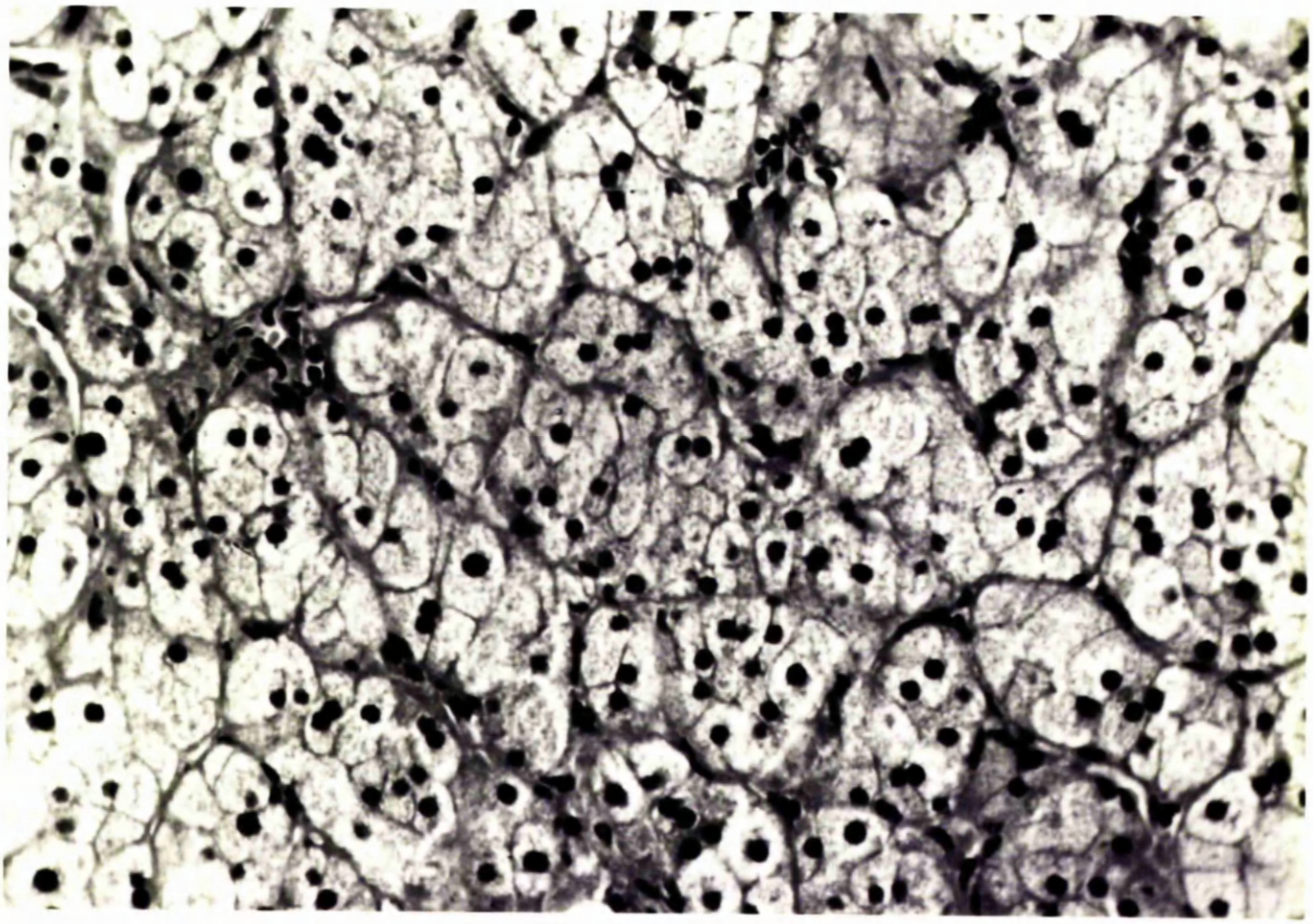


Fig. 36. Histological appearance of one of the adrenal cortical adenomas studied here (Case 1 in Fig. 38). Stained by H. and E. x 400.

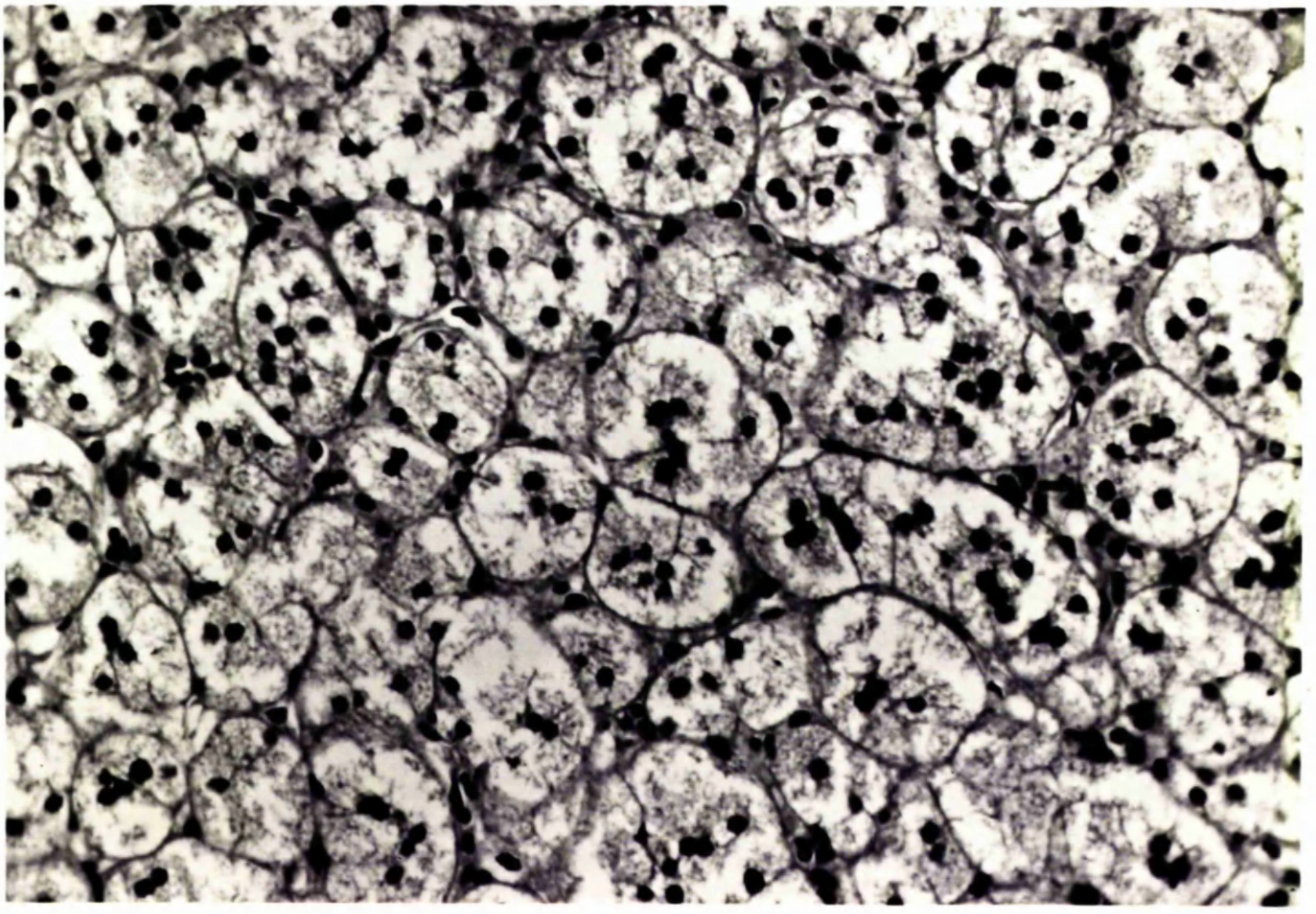


Fig. 37. Histological appearance of one of the adrenal cortical adenomas studied here (Case 2 in Fig. 38). Stained by H. and E. x 400.

nodules varied in weight from 300 mg. to 6 g. In spite of the fact that in four cases the patient was pretreated with commercial ACTH for several days, all nodules were yellow, and histological examination showed that they consisted of lipid laden cells typical of the normal unstressed zona fasciculata (Symington, 1960). Two typical examples of the histological appearances are shown in Figs. 36 and 37. Fig. 38 shows the output *in vitro* by slices of these adenomata in two typical experiments compared with slices cut from the normal cortex of the glands bearing the adenomata. It may be seen, contrary to expectations based on *in vivo* behaviour of these adenomata, that the output by the cells from the adenoma is equal to, or even slightly exceeds, the output of steroids by the remainder of the cortex. This has been found in all six cases studied irrespective of *in vivo* treatment of the patient. Paper chromatography showed that the proportion of cortisol to corticosterone was exactly the same as that produced by the neighbouring cortex, namely approximately 5:1, and no unusual steroids were detected in the chromatography system employed.

8. Effect of *in vivo* ACTH on subsequent *in vitro* steroid production.

Fig. 39 shows that glands pretreated *in vivo* by large doses of corticotrophin do not secrete as much corticosteroid *in vitro* as glands which were not so treated. Both glands were obtained from the same patient, the second after four days of intramuscular corticotrophin. The *in vitro* steroid output from the second gland was

less than half that obtained previously from the first gland. This was found to be true in two similar further experiments.

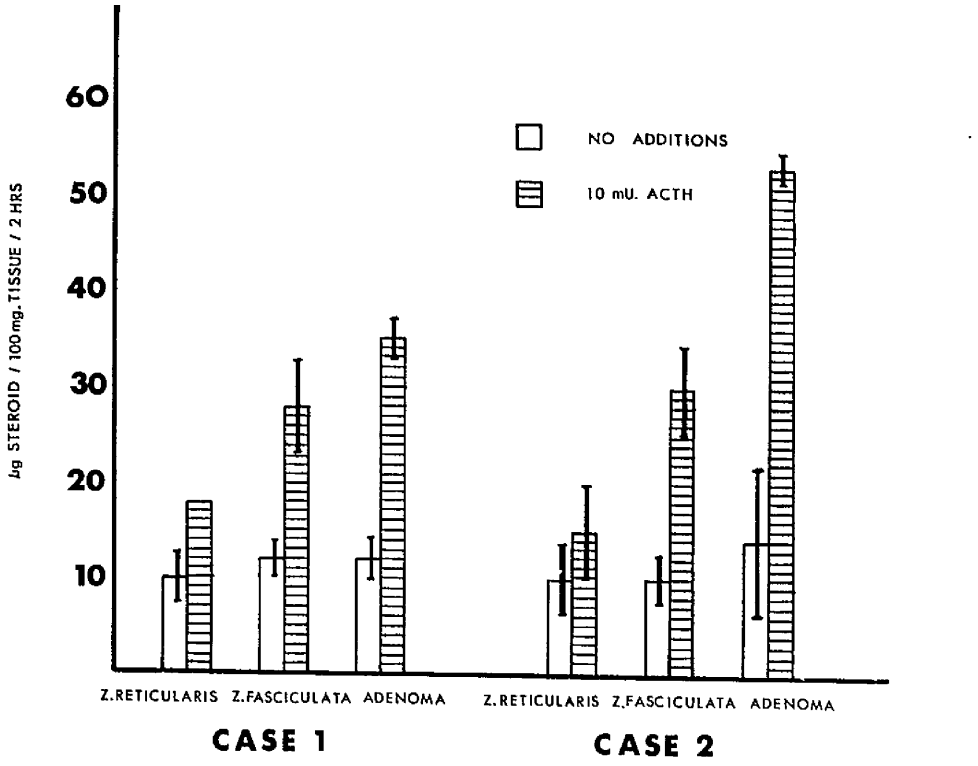


Fig. 38. Output of steroids by slices from various regions of human adrenal glands which contained yellow cortical adenomata.

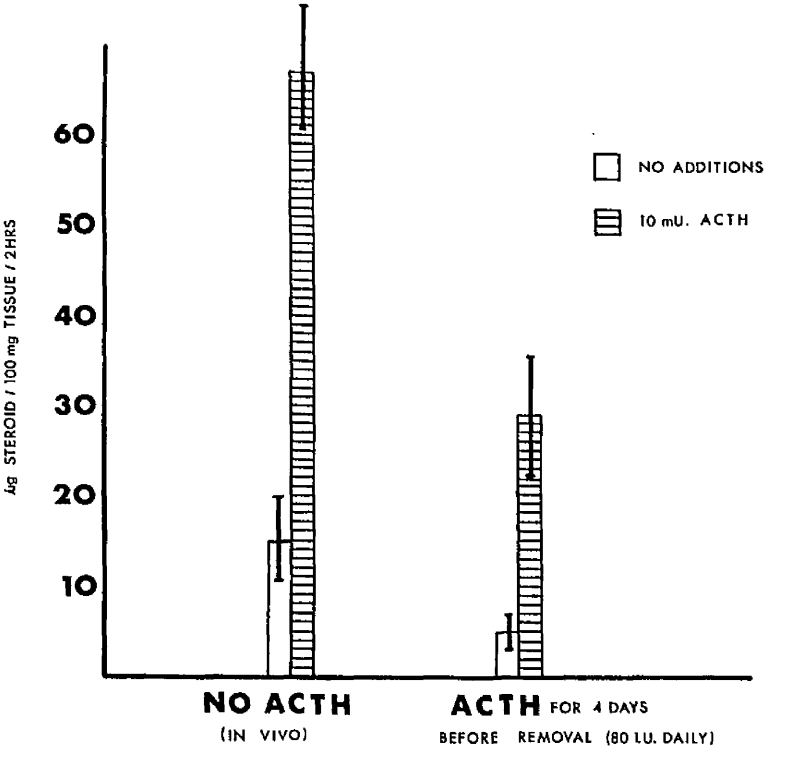


Fig. 39. Output of steroids by slices of the zona fasciculata of the human adrenal cortex. The experiments shown are on two glands obtained from the same patient at an interval of 7 days. When the patient was treated preoperatively with crude ACTH the subsequent response <u>in vitro</u> to corticotrophin is less marked.

DISCUSSION

It is a common feature of the experiments presented in this section that where a difference in steroid output *in vitro* in response to corticotrophin has been found between any two types of cell, those cells differ in their stainable lipid content. Thus, cells in the adrenal cortex from cases of Cushing's Syndrome studied here contained less than the usual amount of stainable lipid - so *in vitro* they responded to corticotrophin with lower corticosteroid output than the normal glands. Similarly the glands which were depleted of stainable lipid by *in vivo* administration of corticotrophin had produced less steroid in response to corticotrophin *in vitro* than the stimulated adrenals. On the other hand the adrenal adenomata studied here contained abundant intracellular lipid, and their *in vitro* steroid was very high.

The *in vitro* results of Griffiths (1960), confirmed here, which showed a much more marked response to corticotrophin of the zona fasciculata than of the zona reticularis, were explained by him in this way. Griffiths analysed his slices for cholesterol, and found that the zona fasciculata has a much higher cholesterol content than the zona reticularis, as is to be expected on the basis of histochemical examination of the whole cortex. Thus all these findings lead to a generalisation that the response to corticotrophin of the cells of the human adrenal cortex *in vitro* depends solely on the lipid content of

the cells. The lipids either constitute, or are associated with, ready precursors for corticosteroid secretion, and these precursors are presumably cholesterol esters or free cholesterol.

It must be emphasize , however, that these findings apply only to *in vitro* conditions. No inference can be drawn as to what actually happens under normal conditions from such *in vitro* studies, but the system does show potentialities of the incubated cells. For instance, zona fasciculata responds to corticotrophin more markedly *in vitro* than zona reticularis - this merely shows that under optimum conditions these cells are capable of very marked sudden secretion of steroids, but does not imply that the zona fasciculata is the chief site of normal production of corticosteroids. Similarly, the adenomata studied here were capable of very marked steroid production under optimum conditions of oxygenation and corticotrophin availability and yet there is good indirect evidence that these nodules were nonfunctional *in vivo*: the patients had no endocrine symptoms and administration of crude ACTH had no effect on the histology of the adenoma, while causing lipid depletion in the neighbouring cortical tissue. It is clear, therefore, that some factor must operate in the gland under normal conditions *in vivo* to prevent the adenomata from losing their lipid. This may mean that they were not exercising their full capabilities for steroid secretion, but it is at present impossible to prove this point.

Comparison of the histological appearances and enzyme activities before and after incubation indicates that the conditions employed do not ensure complete cell survival, particularly of the clear cell which shows the greatest potential for *in vitro* corticosteroid output. Some of the cells are obviously necrotic, other cells show marked degenerative changes. Nevertheless, corticosteroid output has continued and the response to corticotrophin can be elicited for as long as nine hours of incubation. It seems therefore that while dead cells (e.g., from post-mortem gland) or cells poisoned with cyanide cannot secrete corticosteroids, optimum conditions and full cell survival are not essential for this process. While the secretion of corticosteroids is a highly specialised function of the adrenocortical cell, it is also one of its hardier properties.

This finding makes it likely that under *in vitro* conditions the corticosteroid output involves only the final stages of corticosteroid biosynthesis i.e., side chain cleavage and hydroxylation of cholesterol. These processes are known to require molecular oxygen, and this would explain complete inhibition by cyanide, since cyanide is known to inactivate cytochrome oxidase. $NADPH_2$ is necessary and when its production is inhibited by 2-AMP, corticoid production is inhibited to the same extent. Thus cholesterol or its esters may be regarded as a store of readily available hormone precursor, and the process of steroid release may be conceived to include rapid molecular alterations

from cholesterol to fully hydroxylated steroids. This situation is perhaps analogous to storage of zymogen granules in the cells of exocrine glands, where the release of the secretory product also involves its conversion into the active form of the enzyme.

In the experiments in which 2-AMP was added together with corticotrophin to slices of human adrenal cortex, it was necessary to add 120 µmoles of 2-AMP to achieve 40% inhibition of corticoid production (Fig. 29), while in homogenates reduction of NADP was inhibited to the same extent by only 8 µmoles of 2-AMP in the same volume of medium (Table XXVI). This difference is most likely due to the low permeability into the intact cell of the highly charged 2-AMP molecule, and it was found by Leibman and Heidelberger (1955) that isotopically labelled 2-AMP has very low permeability into intact mammalian cells. They found that the intracellular concentration of 2-AMP was only 5% of its concentration in the external medium. If similar factor for permeability of 2-AMP applies to the human adrenocortical cell, the degree of steroid inhibition caused by 120 µmoles of 2-AMP can be explained by the inhibition of the production of $NADPH_2$. This illustrates again the importance of this coenzyme for steroid production by the human adrenal cortex.

The finding that freezing and thawing of the tissue abolishes the response to corticotrophin was discussed by Koritz and Peron (1959). It is clear that intact intracellular organisation is essential for

the response of the adrenal cell to corticotrophin. This effect is perhaps related to the well known lack of response to corticotrophin of tissue homogenates and other broken cell preparations. Freezing and thawing is a less drastic procedure than the methods used to prepare cell homogenates, and the damage is probably limited to intracellular membranes such as the mitochondrial membrane. Several key enzymes are located in the mitochondria, for example, the enzyme which splits off the side chain from cholesterol, while cholesterol and $NADPH_2$ (a necessary cofactor for this reaction) are present principally in the cell sap. If one aspect of corticotrophin action is to facilitate the contact between the enzyme, the substrate and the cofactors (Saba, 1960), damage to a restraining surface, such as the mitochondrial membrane, would result in higher corticoid output. However, lack of response to corticotrophin would be expected, and this is in fact the finding after freezing and thawing of slices.

The marked sensitivity of the human adrenal cortex cells to corticotrophin is of importance in the evaluation of the conflicting results reported for the blood corticotrophin levels found in Cushing's Syndrome. Davies and her colleagues (1960) found that the normal blood corticotrophin level is approximately 0.7 mU/100 ml. blood but in Cushing's Syndrome the level is approximately three times this value. Liddle and Williams (1962) found corticotrophin levels in surgically treated patients with Cushing's Syndrome to be 3-60 mU/100 ml.

blood but they extrapolate their findings to include all cases of Cushing's Syndrome, and such high blood levels have been confirmed by Clayton (see Symington, 1962). It might be wondered therefore, whether the low levels found by Davies are sufficient to account for increase in steroid production seen in Cushing's Syndrome. The data presented in Fig. 26 show that this is indeed the case. The normal blood corticotrophin level found by Davies and colleagues, 0.7 mU %, corresponds to 0.01 mU/1.5 ml., and this concentration gives appreciable response in the *in vitro* system. The levels seen in Cushing's Syndrome, approximately 2 mU %, correspond to 0.03 mU in 1.5 ml., and this falls on the linear part of the response of adrenal cortex to corticotrophin (Fig.26) and thus would cause a considerable increase in corticosteroid output. Levels higher than 7 mU % would probably have no additional effect on the adrenal cortex, as this is the concentration at which maximal response is seen *in vitro*. Thus the levels of corticotrophin found by Davies and colleagues in the blood of patients with Cushing's disease seem sufficient to account for the high corticosteroid production.

In the experiments reported here, little attention was paid to the steroid output in the period during which corticotrophin was not added. The values obtained in this period are of lower accuracy than those seen after corticotrophin addition, since the amounts secreted by 50 mg. of tissue in 2 hours without corticotrophin is close to the

limit of detection of steroids using the method employed here, so, no attempt was made to attach any significance to the variation in output noted. In addition, the results illustrated in Fig. 27 show the persistence of the stimulatory effect of corticotrophin. It seems that corticotrophin is fixed in the cells for some time, or alternatively that its continued presence is not essential, its function being to initiate a train of events which then continues by its own momentum for some time. Thus the stimulation by the endogenous corticotrophin at the time of removal of the adrenal from the body will probably be the determining factor on the levels of steroids secreted *in vitro* without corticotrophin addition.

For this reason, and because the incubated adrenal tissue is undergoing early degenerative changes, the attempts of Byrenforth and her colleagues (1960) and of Bailey *et al.*, (1960) to correlate secretory patterns of adrenal tissue *in vitro* with clinical and pathological diagnosis could not be expected to lead to clear cut correlations. These workers found, however, that human adrenal tissue releases hormones in patterns resembling those secreted by normal tissue *in vivo*, the principal corticosteroids being cortisol and corticosterone, in proportion of 5 to 1. These findings have been confirmed here.

Bailey and co-workers (1960) have included three adrenal adenomata in their *in vitro* studies. They give no histological description

of these adenomata but the weight was similar to the adenomata studied here. However, these adenomata gave rise to clear symptoms of primary aldosteronism and their removal was followed by remission of clinical features. Bailey and colleagues found that these adenomata secreted more corticosteroids *in vitro* than the adjacent adrenal tissue, but the findings reported here show that this is also a feature of adenomata which do not give rise to symptoms. Thus the generalisation was made that the response *in vitro* depends on the contents of the precursors in the cells but has little physiological or pathological significance.

CONCLUSIONS

1. *In vitro* secretion of corticosteroids by slices of human adrenal cortex has been studied and found to have many of the characteristics which have been described for rat adrenal tissue. However, the human adrenal cell is approximately 1000 times as sensitive to corticotrophin as the rat adrenal cell.

2. Although human adrenal cortex slices secrete corticosteroids and respond to corticotrophin *in vitro* many cells within the slices undergo degenerative changes and necrosis during the incubation.

3. Inhibition of NADP linked dehydrogenases by 2-adenylic acid results in decreased corticosteroid output by human adrenal slices.

4. The cyclic nucleotide 3',5'-AMP has the same effect on corticosteroid output by human adrenal tissue as corticotrophin. Insulin and vasopressin have no effect.

5. Slices from glands obtained from patients with Cushing's Syndrome produce rather less corticosteroid in response to corticotrophin than slices from glands obtained from normal patients. On the other hand slices from adenomata which gave rise to no symptoms *in vivo*, produce rather more corticosteroid than nontumourous tissue under *in vitro* conditions.

6. The generalisation was made that under in vitro conditions the corticosteroid output in response to corticotrophin depends solely on the cell content of the easily available precursors for corticosteroids production.

GENERAL SUMMARY

SUMMARY

Studies made on the adrenal cortex of glands removed by operation on women with breast carcinoma indicate that the effects of preoperative administration of corticotrophin depend to a large extent on the degree of purity of the corticotrophin preparation used. Crude preparations of corticotrophin cause a marked increase in adrenal weight and mitotic activity and result in the striking alteration of histological appearances of the adrenal cortex previously described by Symington et al., (1956). On the other hand, administration of corticotrophin concentrated by oxycellulose adsorption and further purified by column chromatography leads to a slight increase in adrenal weight and mitotic activity and causes less alteration in histological appearances of the adrenal cortex.

These findings appear to support the view of Stack-Dunne and Young (1954) that there may be more than one pituitary factor with effects on the adrenal cortex.

Measurements of the weight of 30 adrenal glands removed by operation from these patients gave a mean weight of 4.0 g. This is the first reported series of observations on the weight of adrenal gland in living subjects who were not preoperatively treated by corticotrophin or cortisone.

Administration of all types of corticotrophin to patients who were adrenalectomised four days later show a marked increase in

activity of an enzyme of the pentose phosphate cycle, (the glucose-6-phosphate dehydrogenase). Other dehydrogenases which reduce nicotinamide-adenine dinucleotide phosphate were also studied but do not show significant increases in man, though they do increase in activity after corticotrophin administration in the rat and the guinea pig adrenal cortex.

The increase in glucose-6-phosphate dehydrogenase noted in the human adrenal cortex is of the same magnitude whether adrenal growth is slight or intense, and is maximal in the region of the cortex which corresponds to the interface between the "clear" and "compact" cells. These cells normally are typical of the zona fasciculata and zona reticularis, respectively.

The above findings are discussed in relation to the theory of the mechanism of corticotrophin action proposed by Haynes, and are taken to give this theory further support.

The <u>in vitro</u> response to corticotrophin of slices of human adrenal cortex removed by operation was also studied, and some properties previously found to characterize the adrenal tissue of the rat under <u>in vitro</u> conditions are found to apply to human adrenal tissue. In particular, it was demonstrated that adenosine-3',5'-cyclic monophosphate can stimulate corticosteroid output to the same extent as corticotrophin. Human adrenal tissue, however, was found to be 1000 times more sensitive to corticotrophin than rat adrenal tissue.

It was also noted that in-vitro incubation does not ensure complete cell survival in the adrenal slices; clear cells are more vulnerable than compact cells.

Slices obtained from adrenal glands removed from patients with Cushing's Syndrome or from patients receiving corticotrophin pre-operatively, responded to corticotrophin less well than those from normal glands. On the other hand, slices cut from the so called non-functioning nodules found in the adrenals of patients with no endocrine symptoms, responded in vitro to corticotrophin with higher corticosteroid production than was observed when slices from normal glands were so treated.

It is concluded that secretion of steroids by human adrenal cortex in vitro in response to corticotrophin, does not represent the truly physiological process taking place under the normal conditions, but depends entirely on the conversion of easily convertible stored precursors, and the magnitude of the response depends entirely on the quantity of the precursors which happen to be present in the cells at the time of the removal from the body.

R E F E R E N C E S

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SUMMARY

Studies made on the adrenal cortex of glands removed by operation on women with breast carcinoma indicate that the effects o preoperative administration of corticotrophin depend to a large exte on the degree of purity of the corticotrophin preparation used. Crude preparations of corticotrophin cause a marked increase in adre weight and mitotic activity and result in the striking alteration of histological appearances of the adrenal cortex previously described by Symington et al., (1956). On the other hand, administration of corticotrophin concentrated by oxycellulose adsorption and further purified by column chromatography leads to a slight increase in adre weight and mitotic activity and causes less alteration in histologic appearances of the adrenal cortex.

These findings appear to support the view of Stack-Dunne and Young (1954) that there may be more than one pituitary factor with effects on the adrenal cortex.

Measurements of the weight of 30 adrenal glands removed by operation from these patients gave a mean weight of 4.0 g. This is the first reported series of observations on the weight of adrenal gland in living subjects who were not prooperatively treated by corticotrophin or cortisone.

Administration of all types of corticotrophin to patients who were adrenalectomised four days later show a marked increase in

activity of an enzyme of the pentose phosphate cycle, (the glucose-6-phosphate dehydrogenase). Other dehydrogenases which reduce nicotinamide-adenine dinucleotide phosphate were also studied but do not show significant increases in man, though they do increase in activi after corticotrophin administration in the rat and the guinea pig adrenal cortex.

The increase in glucose-6-phosphate dehydrogenase noted in the human adrenal cortex is of the same magnitude whether adrenal growth is slight or intense, and is maximal in the region of the cortex whi corresponds to the interface between the "clear" and "compact" cells These cells normally are typical of the zona fasciculata and zona reticularis, respectively.

The above findings are discussed in relation to the theory of the mechanism of corticotrophin action proposed by Haynes, and are taken to give this theory further support.

The <u>in vitro</u> response to corticotrophin of slices of human adre cortex removed by operation was also studied, and some properties previously found to characterise the adrenal tissue of the rat under <u>in vitro</u> conditions are found to apply to human adrenal tissue. In particular, it was demonstrated that adenosine-3',5'-cyclic monophosphate can stimulate corticosteroid output to the same extent as corticotrophin. Human adrenal tissue, however, was found to be 1000 times more sensitive to corticotrophin than rat adrenal tissue.

It was also noted that in-vitro incubation does not ensure complete cell survival in the adrenal slices; clear cells are more vulnerabl than compact cells.

Slices obtained from adrenal glands removed from patients with Cushing's Syndrome or from patients receiving corticotrophin pre-operatively, responded to corticotrophin less well than those from normal glands. On the other hand, slices cut from the so called non-functioning nodules found in the adrenals of patients with no endocrine symptoms, responded <u>in vitro</u> to corticotrophin with higher corticosteroid production than was observed when slices from normal glands were so treated.

It is concluded that secretion of steroids by human adrenal cortex <u>in vitro</u> in response to corticotrophin, does not represent th truly physiological process taking place under the normal conditions but depends entirely on the conversion of easily convertible stored precursors, and the magnitude of the response depends entirely on the quantity of the precursors which happen to be present in the cel at the time of the removal from the body.