STEROID PRODUCTION BY INCUBATED MOUSE ADRENALS

By Henry Triller

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Allan Memorial Institute of Psychiatry
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ABSTRACT

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Adrenals from adult male mice, when incubated in Krebs-Ringer bicarbonate glucose solution, produce corticosterone, aldosterone, and a polar lipid with the properties of 18-hydroxy-corticosterone; the polar fraction also contains other reducing lipids. No evidence of 17-hydroxylated compounds or of 18-hydroxy-11-deoxycorticosterone was obtained. ACTH stimulates most the production of corticosterone, it also affects the other two fractions but to a much lesser degree. ACTH-stimulated steroid production decreases with time; the unstimulated production remains constant for as long as ten hours. The response to ACTH is greatly increased by calcium. The dose-response curve to ACTH is linear between 0.1 and 1.0 U of ACTH per 100 mg adrenal weight and logarithmic between 1.0 and 100 U. The mouse adrenal is more responsive to ACTH than the rat adrenal. The mouse adrenal does not differentiate between concentrations of ACTH in solution, only between total amounts.

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I. INTRODUCTION

(A) The Adrenal Gland Throughout the Vertebrate Kingdom

1. Early History

The adrenal glands were first described in 1563, when at that time Bartholomaeus Eustachius noted them as "Glandulae renibus incumbentes". Since then, they have gone under several names; "capsulae renales", "glandulae renales", "capsulae atrabilariae" and the more familiar name of suprarenal capsules. In the 17th century the prevalent idea was that the adrenals were functional only in foetal life.

In that early period of biological research it was difficult to conceive of ductless organs that could elaborate materials for discharge into blood, and it was not until Claude Bernard that the idea of internal secretion became accepted (1).

2. Anatomy of the Adrenal Gland

The location and macroscopic appearance of the adrenal depends on the relationship which the adrenal cortex bears both to chromaffin tissue and to the kidney (2). The chromaffin tissue consists of cells derived from the ectoderm, and the cortical tissue is composed of cells derived from the mesoderm (1).

Jones et al. describe the changes occurring in the anatomy of the adrenal through evolution of the vertebrates (2). The basic unit of cortical tissue is a cord of cortical cells. In cartilaginous fish,

cortical cords are joined together to form an interrenal gland, lying posteriorly on or between the kidneys. Chromaffin tissue is completely separate. In bony fish, the cortical cords lie embedded in the anterior or head-kidney which represents a non-functional, degenerated kidney. The cortical cords are formed into groups of different sizes and distribution, usually associated with chromaffin cells (2).

In amphibians, adrenocortical tissue is visible as discrete or scattered areas lying ventrally on the mesonephros (2). It comprises cortical cords running mainly longitudinally. Chromaffin cells are found amongst the groups of cortical cells. The whole gland is contained peripherally by connective tissue but centripetally merges with the kidney.

The adrenal gland of reptiles consists of cortical cords running chiefly longitudinally, as in amphibians. Islets of chromaffin tissue intermingle with the adrenal cortex. The bird adrenal is more compact than that of the reptile, with a firmer outer connective tissue capsule, against which the cortical cords loop, thus providing the first indication of cortical zoning (2). In marsupials and eutherian mammals the cortical cords are placed between a firm, well-developed outer connective tissue capsule and centripetally against the central medulla, into which the chromaffin tissue has coalesced.

It seems that in evolution, the chromaffin tissue, at first separate, becomes intermingled with cortical tissue, forming islets and finally coalesces as the medulla, a central mass in the adrenal glands of

mammals. At the same time, these scattered cortical and chromaffin cells are very closely associated with the renal system. However, starting with the reptiles, the chromaffin and cortical tissue were formed into a discrete encapsulated mass, vascularized separately from the renal system, and the adrenal gland as such was formed. In most mammalian species the cortex represents 85-90% of the whole adrenal gland (1).

3. Histology of the Adrenal Cortex

The adrenal glands of the mammals are paired structures lying in the region of the anterior pole of the kidney. Connective tissue surrounds the adrenal glands and provides an internal supporting framework.

Three main concentric zones may be distinguished in the mammalian adrenal cortex, upon histological examination: the zona glomerulosa, lying against the outer connective tissue capsule, the zona fasciculata which consists of long columns of cells running centripetally and the zona reticularis surrounding the medulla (1). Sometimes an extra zone is observed, called the zona intermedia. It consists of narrow cuboidal cells with small, darkly staining nuclei and lies between the zona glomerulosa and the zona fasciculata (1). The zona glomerulosa, characteristic of the mammals only, arrived late in evolution.

In the loose and unrestricted adrenals of the lower vertebrates, the cells are difficult to differentiate on histological grounds. However,

this differentiation exists as demonstrated by the effects of hypophysectomy, which causes complete atrophy of some cortical cells, while others are of normal appearance.

4. Evidence that the Adrenal is Essential for Life

In 1856 Brown-Sequard, the French physiologist, reported that dogs, cats, and rabbits died twelve hours after adrenalectomy (3). This was the first proof that the adrenal gland was essential for life.

In 1895 Oliver and Schäfer (4) announced the discovery of a potent pressor substance in aqueous adrenal extracts. They later showed that the active principle is produced in the medulla and not in the cortex. Thus it was established that the two anatomical divisions of the adrenal have distinct functions.

5. Evidence that the Life-Maintaining Principle is Derived from the Cortex

Because of the strong physiological activity of the medullary substance, it was assumed that death in adrenalectomized animals was due to lack of the pressor activity. In 1913 Biedl (5) showed that the destruction of the medulla had little effect on dogs and rabbits, whereas death always followed removal of the cortex, even if the medulla remained intact. Animals would live with only 1/8 of their adrenal tissue, provided it was cortical tissue. This was the first evidence that the factor essential for life resides in the cortex.

6. The Manner in Which the Cortex Exerts Its Influence

Adrenalectomy produces a fall in the plasma levels of sodium and chloride and a corresponding rise in the plasma potassium levels (6). Adrenalectomized animals can be kept alive by injection of sodium salts (7) or by dietary administration of sodium chloride (8,9). This indicated that the major life-maintaining action of the adrenal cortex is related to its ability to regulate sodium metabolism. Adrenalectomy also causes a severe disturbance in the carbohydrate metabolism, apparent from a fall in blood sugar and a depletion of the glycogen stores of the liver, heart and muscle. This effect is largely due to decreased gluconeogenesis (10).

It was observed that the adrenal produces two groups of compounds, one exerting its influence on carbohydrate metabolism (11, 12), the other affecting mainly the electrolyte balance of the body (13).

7. Chemical Structure of the Active Compounds

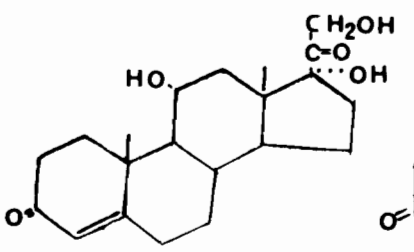
By 1943, twenty-eight steroids had been isolated in crystalline form from adrenocortical tissue (14). Of these, only six were biologically active. They were shown to consists of corticosterone and its derivatives, and were named accordingly: corticosterone, 11-dehydrocorticosterone, 11-deoxycorticosterone, 17α-hydroxycorticosterone, 17α-hydroxy-11-dehydrocorticosterone, and 17α-hydroxy-11-deoxycorticosterone. In 1954 a further active adrenal steroid hormone was identified. It was shown to possess the structure of corticosterone plus an aldehyde function

at C-18 (15) and it was named aldosterone. The structural formulae of these seven compounds are shown in Fig. 1. Aldosterone can exist in the free form, in the hemi-acetal form and in a third form, in which, in addition to the hemi-acetal linkage, a second oxygen bridge is present between C-18 and C-20.

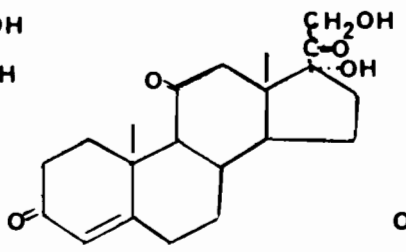
8. Type of Steroid Compounds Produced by the Adrenal Cortex

As stated previously, the adrenal synthesizes glucocorticoids which influence carbohydrate metabolism, and mineralocorticoids which influence electrolyte metabolism. Glucocorticoid activity seems to be associated with the presence of an oxygen function at C-11, and is further enhanced by an additional oxygen at C-17 (16). Thus cortisol and cortisone are potent glucocorticoids; 11-deoxycorticosterone has very little glucocorticoid activity but causes a great increase in sodium retention by the body. Aldosterone is an even more potent mineralocorticoid than 11-deoxycorticosterone. Most corticoids exhibit both kinds of activity to some extent.

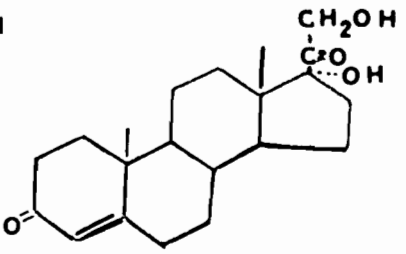
In addition to the adrenocorticoids, the adrenal cortex is able to synthesize sex hormones. Androgens have been identified in the adrenal gland (17) of several species. Estrogens were isolated from beef adrenals (18). Progesterone, a key intermediate in steroid synthesis, was also isolated from adrenal tissue (19). It seems to be a normal secretory product of the adrenal.



17α-Hydroxycorticosterone



17α-Hydroxy-11-dehydrocorticosterone



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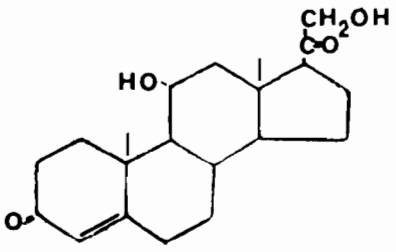
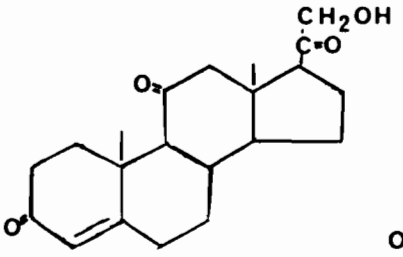
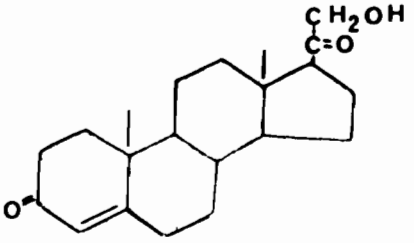


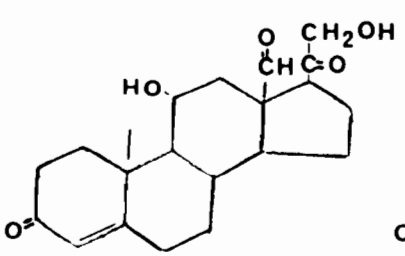
Corticosterone

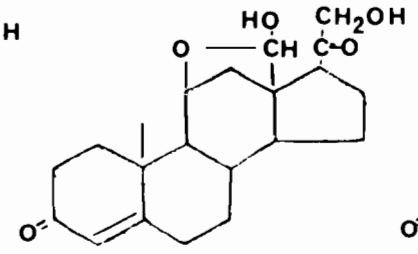


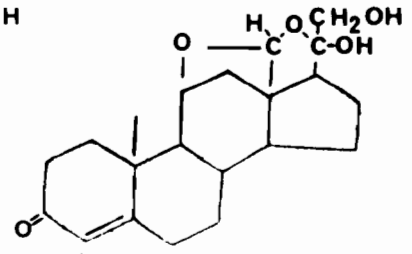
11-Dehydrocorticosterone



11-Deoxycorticosterone







Aldosterone

FIG. 1. The Adrenocorticoids

9. Nature of the Steroids Produced in the Different Cortical Zones

Ayres et al. (20,21) incubated tissue from the three zones of ox adrenals and found that cortisol was produced in the zona fasciculata; aldosterone in the zona glomerulosa and corticosterone in both zones. This confirmed histological evidence that different zones may produce steroids with different biological actions.

Symington and co-workers (22) suggest that the zona fasciculata serves merely as a storage zone for steroid precursors and that the zona reticularis is the actual site of production of C-21 compounds, with the exception of aldosterone.

This would suggest that the electrolyte balance of the body is under the influence of the zona glomerulosa, while carbohydrate metabolism is controlled by the other two zones.

10. Methods Used in Studying the Adrenal Secretory Products

In order to determine whether a gland secretes a compound or merely releases it, the compound must be detected in the venous blood of the gland in greater concentrations than those observed in simultaneously drawn arterial blood. Adrenal vein cannulation, perfusion of the adrenal gland and *in vitro* studies were used to determine the qualitative and quantitative secretions of the adrenal cortex.

Perfusion of the adrenal gland with 11-deoxycorticosterone, a compound of low glycogenic activity, yielded an extract of high glycogenic

activity, indicating oxygenation at the C-11 position (23,24). Levy et al. (25) identified this activity as due to corticosterone. They were also able to demonstrate the conversion of 17α-hydroxy-11-deoxy-corticosterone to cortisol. Further perfusion studies by Hechter and co-workers showed that the adrenal can introduce hydroxyl groups at the C-17, the C-21 as well as at the C-11 positions. These findings gave evidence of active biosynthesis in the adrenal cortex.

The observation that the rat adrenal contains only 2-3 mg of corticosterone per 100 mg of tissue (26,27), while extracts of incubation media yielded 15-20 mg total ultraviolet-absorbing lipids per 100 mg of tissue per 2 hours (28) indicated that corticoids are being synthesized in vitro and not merely released.

(B) Influence of the Pituitary on the Adrenal Cortex

1. Effects of Hypophysectomy

In 1927, Smith (29) developed the first successful method of hypophysectomy in the rat and reported among other symptons a marked atrophy of the adrenal cortex. This atrophy could be reversed by the administration of tissue from the anterior, but not posterior, lobe of the pituitary (30). From these experiments he concluded that the growth of the adrenal cortex is regulated by the anterior pituitary.

Yates and Urquhart reported in their review paper (31) that plasma levels of the adrenocorticoids fall considerably after hypophysectomy and that the secretion rates of cortisol and corticosterone

drop to values that are less than 20% of those in intact animals. Further evidence for a pituitary-adrenal relationship was given by Wilson et al. who observed that mice bearing tumors of the pituitary gland secreted twice as much corticoids as control animals (32). There is some evidence for a species difference in the extent of the regulation of adrenocortical function by the pituitary (33).

2. Isolation of ACTH from Pituitary Tissue

In 1943, two groups of co-workers isolated a homogeneous protein preparation from pituitary tissue of sheep (34) and hog (35) which specifically stimulated the size and output of the adrenal cortex, and was therefore called adrenocorticotropic hormone (ACTH).

With improved methods of purification and isolation of proteins, potent ACTH preparations of high purity became available. ACTH was shown to be a peptide of molecular weight about 5000, containing 39 amino acids. For ACTH activity the first 23 amino acids from the N-terminal are required, the first 13 amino acids give melanocyte-stimulating hormone (M.S.H.) activity. The composition from amino acid 23-39 may vary with different species (36).

3. Adrenocortical Zones Controlled by the Anterior Pituitary

It became evident that the adrenal cortex is not entirely under the control of the pituitary from the fact that the hypophysectomized animal does not exhibit all the symptons seen after adrenalectomy (37,38).

Smith (30) has shown that following hypophysectomy in the rat, atrophy is most marked in the zona fasciculata. Yoffey and Baxter (39) suggested that the zona fasciculata and not the zona glomerulosa is under pituitary control. By administering adrenal cortical extract to rats, they obtained an accumulation of cholesterol throughout the cortex, and upon subsequent administration of ACTH they found that cholesterol had disappeared from the zona fasciculata and the zona reticularis but not from the zona glomerulosa. Stachenko and Giroud (40) incubated beef adrenal slices of the three cortical zones separately, and measured the steroid production with and without the addition of ACTH. They found that ACTH stimulates the steroid production in the zona fasciculata and the zona reticularis, but has little effect on the zona glomerulosa.

These results substantiate the histological findings of Deane and Greep (41,42) who observed that following hypophysectomy the innermost two zones atrophied, with an accompanying disorder in carbohydrate metabolism. The zona glomerulosa, on the other hand, appeared normal and no effect on electrolyte balance was observed. Changes in the dietary sodium-potassium ratio, however, were reflected in the zona glomerulosa but not in the other two zones. They concluded that the carbohydrate controlling principle is in the zona fasciculata, while the salt regulating principle is produced in the zona glomerulosa.

Jones and Roby showed that male mice that have been hypophysectomized for as long as 100 days show no abnormalities in their intake and output of sodium and potassium. The hypophysectomized animal thus can control its water, sodium and potassium metabolism (43).

From these results it has been concluded that ACTH has little or no effect on the zona glomerulosa and consequently on the electrolyte balance of the body. However, ACTH plays a vital role in the elaboration of hormones by the two innermost zones and, in this way, is one of the factors controlling the carbohydrate metabolism of the body.

4. Effects of ACTH on:

a. Adrenal Size

As noted previously, the pituitary controls adrenal size (30). It was thought that the size of the adrenal gland was dependent only on ACTH; however, Lostroh and co-workers showed that the adrenal hypertrophy following ACTH administration in the mouse could be enhanced by the presence of growth hormone, although the latter hormone alone had no effect on adrenal weight (44). These findings suggest that the growth hormone, present as a contaminant, may be responsible for the adrenal hypertrophy following ACTH administration.

b. Adrenal Blood Flow

Balfour (45) found an increase in adrenal blood flow following ACTH administration in very young calves.

c. Oxygen Uptake and Phosphorus Incorporation by the Adrenal Gland

Oxygen consumption is markedly increased in the adrenal following the addition of ACTH in vivo (46) or in vitro (47,48).

Gemzell (49), working with intact rats injected with radioactive phosphorus, demonstrated that ACTH increases the rate of incorporation of P^{22} into organic compounds of the adrenal.

d. Cholesterol Content of the Adrenal

In 1943, Sayers (50) reported that an injection of a hog pituitary preparation produced a 50% reduction in the adrenal cholesterol level of the rat. He showed further (51) that if the pituitary was present, stressing agents were also capable of depleting cholesterol stores of the adrenal. Stress was known to cause an increased production of steroid hormones (52); Sayers concluded that the changes in the cholesterol content of the adrenal, following stimulation by a hog pituitary ACTH preparation, were associated with the utilization of cholesterol in the synthesis of corticoids.

e. Ascorbic Acid Content of the Adrenal

Sayers also reported that ACTH produces a drop in the ascorbic acid levels of the adrenal (53). Various stressing agents are capable of doing this as well (51).

The role of ascorbic acid and the significance of its disappearance in steroid biosynthesis remains unknown. Hechter and Pincus assume it to act by protecting the biosynthetic enzymes from oxidative inactivation (54), while Kersten et al. suggest that ascorbic acid kept in the reduced form by DPNH may function in steroid hydroxylations (55). It is well known that, following the administration of ACTH, the steroid

output of the adrenal is increased while its ascorbic acid content falls. The ascorbic acid stores are not replenished for three hours, whereas the steroid output returns to its previous lower level after one hour. At this point a second administration of ACTH produces, again, a rise in the steroid output, even though the ascorbic acid content of the adrenal is still low (56).

These findings would suggest that steroid biosynthesis is not very closely related to the amount of ascorbic acid present in the adrenal gland.

f. Production of Corticosteroids by the Adrenal

In 1946, Ingle et al. (57) reported that repeated administration of ACTH to rats produced, in addition to adrenal hypertrophy, also hyperglycemia and glucosuria. They therefore suggested that ACTH stimulates the secretion of the 11-oxygenated corticoids, as these are known to be the group of corticoids involved in the regulation of carbohydrate metabolism (16).

Saffran, Elliott and their co-workers (28,58) found a significant increase in the ultraviolet absorbing lipids following the addition of ACTH to incubating adrenals.

The response of incubated adrenal slices to ACTH is not as marked as that of the perfused glands. Maximal stimulation produces a 2- to 4-fold increase in the steroid production of beef adrenal slices (59) as compared to a 5- to 10-fold increase in the steroid production

obtained in perfusion experiments of the same gland (60). In addition, the amount required for maximal steroid production is much greater in the sliced adrenal than in the perfused adrenal (61). It could be that in perfused glands ACTH, through a vascular route, is brought more efficiently into contact with the cellular structures concerned in steroid secretion. Accumulation of steroids in the incubation medium as well as the inactivation of ACTH during incubation may account, in part, for the smaller corticoid production in incubation experiments (62).

It is difficult to detect an effect of ACTH on the release of corticoids into the adrenal vein (63,64), because the gland has already been maximally stimulated by the stress of the operative procedures (54). No ACTH effect was shown with adrenal homogenates. It should be noted that in homogenates, steroid synthesis, in the absence of ACTH, proceeds at a rate comparable to that of slices maximally stimulated with ACTH (60,65,66). A similar increase in steroid production and lack of response to ACTH is observed in slices in which the cell structure has been disrupted by freezing (59).

The various theories that have been elaborated as to the mode of action of ACTH on steroidogenesis will be discussed in the next section.

(C) Mode of Action of ACTH on the Adrenal

1. Site of Action of ACTH in the Biosynthesis of Adrenocorticoids

The small amounts of ACTH necessary to show a measurable effect

on the adrenal cortex, and the chemical similarity between cholesterol and the corticoids, suggested to Sayers et al. (67) that ACTH acts as a catalyst, increasing the rate of reactions converting cholesterol to corticoids.

In perfusion experiments, using radioactive precursors, it was shown (68) that ACTH has little effect on the conversion of progesterone to cortisol and corticosterone, but greatly stimulates the formation of these compounds from cholesterol, indicating that ACTH acts at a step involving the conversion of cholesterol to progesterone. Further support for this assumption was supplied by Ward and Birmingham (69), when they showed that ACTH does not stimulate hydroxylations at C-11, C-18 or C-21.

2. Proposed Theories for the Mode of Action of ACTH

Various theories have been proposed to explain the mode of action of ACTH on steroid biosynthesis in the adrenal cortex.

Strong evidence has been supplied to show that ACTH acts by increasing the rate of conversion of cholesterol to progesterone. That this increase is not evoked by the stimulation of an enzyme system was deduced from the fact that ACTH does not stimulate the steroid production in homogenates (60,65), which should possess the same enzyme systems as the intact cells. Because homogenates produce corticoids, in the absence of ACTH, at a rate comparable to the maximum rate produced by ACTH-stimulated slices, Hayano et al. (70) suggested that ACTH acts by removing, in some way, restraining factors present in intact cells but absent from

homogenates and also from slices that have been frozen.

Hechter, in his discussion on the mode of action of hormones, pointed out that in no circumstances do hormones act on the rate of an enzyme reaction; they exert their activity only on intact cells (71). His group also found that ACTH specifically increased the permeability of adrenal cells to xylose (72). He, therefore, proposed to explain ACTH activity by its influence on cell permeability (73).

2a. Haynes' Theory on the Mode of Action of ACTH

The adrenal cortex is rich in the enzymes of the hexose monophosphate shunt (74,75), a pathway which supplies the tissue with reduced triphosphorpyridine nucleotides (TPNH). Haynes showed that ACTH increases adrenal levels of phosphorylase (76) (an enzyme which breaks down glycogen to glucose-1-phosphate which in turn enters the shunt via glucose-6-phosphate). Sutherland and Rall found that phosphorylase is activated by adenosine-3',5'-monophosphate (3',5'-AMP) (77). This prompted Haynes to measure 3',5'-AMP in the adrenal and he noted that ACTH did produce an accumulation of 3',5'-AMP (78). Further, addition of 3',5'-AMP to incubating rat adrenals stimulated corticoid production (79).

Haynes, therefore, proposed that ACTH acts by providing the TPNH required for steroid synthesis via 3',5'-AMP mediated stimulation of the adrenal phosphorylase (80). Fig. 2 shows the mode of action of ACTH as postulated by Haynes (taken from a review by Saffran on mechanisms of adrenocortical control (81)).

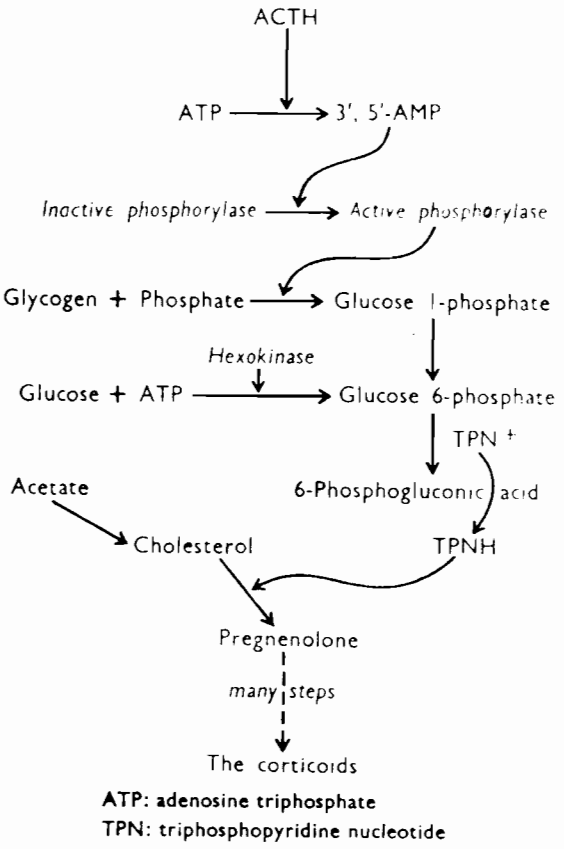


FIG. 2. Proposed mode of action of ACTH on steroidogenesis.

Although Haynes' theory is attractive and explains some observed facts in corticoid synthesis, contradictory evidence has recently accumulated which will be summarized below.

3. Modification of Haynes' Theory

Koritz and Peron (82) showed that adrenal tissue, maximally stimulated by ACTH, can be further stimulated by the addition of TPN^+ and glucose-6-phosphate (G-6-P), and tissue which is maximally stimulated by TPN^+ and G-6-P can be further stimulated by the addition of ACTH. In the latter case, they suggested that the increase in corticoid output upon the addition of ACTH is due to additional precursors made available by ACTH, since there was an excess of TPNH. In the former case, where addition of TPN^+ and G-6-P caused an increased steroid output, they suggested that the amount of precursors was in excess over that of TPNH and that more TPNH was made available from TPN^+ and G-6-P. It should be noted the effect of ACTH + G-6-P + TPN^+ was not additive. These results can be explained by postulating that ACTH, in addition to its effect on the levels of TPNH via phosphorylase, controls the levels of corticoid precursors.

This view is further supported by Koritz and Peron (82). They found that freezing of the adrenal tissue results in a tissue that will not respond to ACTH, but its response to G-6-P + TPN^+ is much greater than the response of normal tissue to maximal stimulation by G-6-P + TPN^+ + ACTH.

They presented the hypothesis that corticoid precursors exist in a bound, enzymatically unavailable form, from which they are released in a controlled manner by ACTH. Freezing does the same but in a non-enzymatic and uncontrolled manner.

4. Deficiencies of the Haynes' Theory

a. At the Phosphorylase Level

Kobayashi et al. (83) showed that the phosphorylase activity decreased linearly with time, and that if they added ACTH to the incubation medium at a time when the phosphorylase activity had disappeared, they still obtained an increase in steroid output. From these experiments, they concluded that phosphorylase does not play an integral part in the increased steroid production by ACTH.

Ferguson (84) showed that puromycin inhibits steroid synthesis in response to ACTH. As puromycin did not inhibit steroid synthesis by a TPNH generating system, and did not inhibit phosphorylase activation by ACTH, he concluded that puromycin must exert its inhibitory influence on a step other than phosphorylase activation.

If Haynes' theory is correct, the glycogen content of the adrenal should be depleted by ACTH. Birmingham found no correlation between the adrenal response to ACTH and the glycogen levels in the adrenal (unpublished data).

b. At the TPNH Level

Harding and Nelson observed, in hypophysectomized rats, a 12-fold decrease in the steroid secretion rate of the adrenals half an hour after the operation. However, the level of TPNH in the adrenal gland remained constant for 72 hours after hypophysectomy (85). They therefore concluded that the cellular concentration of TPNH is not vitally related to corticosteroidogenesis.

They were further supported in their conclusion by the following observation: glucose-6-phosphate dehydrogenase and phosphogluconic acid dehydrogenase (TPNH generating enzymes of the pentase shunt) undergo a loss of activity after hypophysectomy. However, this loss occurs much later than the observed fall in the steroid secretion rates of the adrenal (86). This loss of activity is secondary to some earlier event which caused the decrease in corticosteroidogenesis.

5. Present-Day Knowledge on the Mode of Action of ACTH

It is unlikely that all the effects of ACTH could be explained by a simple theory. It is probable that all the theories put forth are correct to some extent.

The stimulation of corticoid production by 3',5'-AMP cannot be further enhanced or increased by ACTH (87), suggesting that ACTH and 3',5'-AMP act by a common mechanism. Therefore, that part of Haynes' theory which states that ACTH acts via 3',5'-AMP is probably correct. However, it appears unlikely that 3',5'-AMP affects steroid production by activation of phosphorylase. ACTH must act in more than one way, and

it may supply precursors as suggested by Peron. It is doubtful whether phosphorylase and TPNH exert the essential roles, in the adrenal response to ACTH, assigned to them by Haynes.

Birmingham et al. (88) have shown that calcium in the medium is essential for adrenal response to ACTH. Calcium also doubles the steroid output in response to 3',5'-AMP (87). It may be that calcium, in the medium, facilitated access of ACTH to the cellular mechanisms involved in steroid production, because it is known that calcium affects the permeability of cell membranes.

It appears that calcium may link Hechter's membrane theory with Haynes' theory of ACTH action via 3',5'-AMP. It is quite possible that the membrane theory of Hechter and Haynes' theory may merge one day.

(D) Species Differences in the Secretion of Corticoid

The most primitive of vertebrates have been shown to secrete adrenocorticoids. By studying cortical secretions of different species, one obtains more information about the adaptations that animals had to make throughout evolution.

1. Corticoid Secretions in Fish

The fish forms a primitive interrenal gland joined to the kidney, and yet there is abundant evidence that steroid hormones are formed by this gland. Chavin and Kovacevic (89) found that the adreno-

cortical tissue of the goldfish is similar, histochemically, to the zona fasciculata and the zona reticularis of the mammalian adrenal cortex.

Salmon, during spawning, contain high amounts of cortisone, cortisol and corticosterone as well as detectable amounts of aldosterone (90). Incubated interrenal tissue of the dogfish, the skate and the ratfish produce aldosterone (91). In addition, the dogfish and the skate produce small amounts of corticosterone, while cortisol is found in the ratfish.

2. Corticoid Secretion in Amphibians

The adrenal cortex of amphibia merges on the inside with the kidney and there is no zonation of the cortex.

Carstensen et al. (92) reported that the adrenal of the American bullfrog produces mainly aldosterone and corticosterone, the ratio of aldosterone to corticosterone was 4:1, and ACTH stimulated the production of aldosterone much more than that of corticosterone. Kraulis and Birmingham (93) reported the conversion, in frog adrenals, of 11β-hydroxyprogesterone to corticosterone and to fractions with the mobility of aldosterone and 18-hydroxycorticosterone. In bullfrog adrenals, radioactive progesterone was shown to be converted to aldosterone and 18-hydroxycorticosterone (94). Recently, 17-hydroxylated steroids have been reported (95) in the adrenal secretions of two amphibians, Xenophus laevis and Bufo bufo.

3. Corticoid Secretions in Reptiles

The turtle has been reported to produce aldosterone and corticosterone as the major steroid hormones (96). Sandor et al. showed that, in addition to aldosterone, 18-hydroxycorticosterone and 11-deoxycorticosterone are synthesized by turtle adrenals (97). Adrenal glands of lizards and snakes are able to convert progesterone to aldosterone, corticosterone and an unknown steroid (98). This process is not affected by ACTH.

4. Corticoid Secretions in Birds

In the chicken and the duck, 18-hydroxycorticosterone, aldosterone and corticosterone were found to be the major secretory adrenal products (99). Others showed that corticosterone is the major steroid hormone in the chicken, the pigeon, the western gull and the duck. Aldosterone and 11-dehydrocorticosterone were also present in all four species (100). The glands of the chicken and the duck were more responsive to ACTH than those of the pigeon and the gull. Corticosterone was the only compound produced in increased amounts by ACTH.

5. Corticoid Secretions in Mammals

As the adrenal cortex of the mammals is the most specialized, one may expect to observe a great diversification in the cortical secretions of the mammals. For example, the adrenals of the Mongolian gerbil secrete cortisol and 19-hydroxy-11-deoxycortisol, as well as aldosterone (101), and provides the first evidence for hydroxylation at C-19, an unusual position for a hydroxyl group.

The cortical secretions of many mammalian species have been investigated. Those of the less common species will be discussed first. Cortisol is the main plasma corticosteroid of the brush-tailed possum (102). The sea lion produces mostly cortisol and corticosterone (103) as well as small amounts of aldosterone. In the camel, cortisol and corticosterone are the main steroids produced by the adrenal cortex (104); a compound with the mobility of 18-hydroxy-11-deoxycorticosterone was also isolated from camel adrenals. Cortisol has been reported to be the main cortical hormone of sheep, but small amounts of corticosterone have also been detected (105,106). This was also the case in foetal sheep near term (107), indicating that the foetal adrenal is active, at least near term. Sandor and Lanthier (108) reported that the zona glomerulosa of the beef adrenal is capable of converting progesterone to 18-hydroxycorticosterone; 17-hydroxylated steroids, however, make up the major part of beef adrenal secretions (104). Cortisol seems to be the main corticosteroid of the guinea pig adrenal (26,110). Cortisol, corticosterone and small amounts of cortisone constitute the adrenocorticoid secretions of the cat (26). The dog secretes mainly cortisol (26,111,112), but also detectable amounts of corticosterone. Cortisol is also the main adrenal secretory product of the golden hamster (113).

In man, cortisol is the major steroid produced (114,115,116). Corticosterone is the next most abundant compound (114,115). Progesterone was shown to be converted to 18-hydroxycorticosterone in human adrenals (108), and was reported to be present in larger amounts than

aldosterone (117). By the use of sensitive methods, minute amounts of various steroids have been isolated from the human adrenal or from its venous blood.

Cortisol has been shown to be the main cortical hormone of the monkey. Small amounts of corticosterone were also detected (26, 118). Rabbit adrenal cortex secretes corticosterone as the main adrenocorticoid (119,120).

5a. Corticoid Secretion in the Rat

As the rat is a very popular laboratory animal, its corticoid secretion has been investigated by many. Despite early reports that the rat secretes 17-hydroxylated steroids, the major evidence indicates that no such compounds are produced by the rat adrenal.

Ward and Birmingham (121) confirmed the findings of other workers that corticosterone is the major secretory product of the rat adrenal and that aldosterone is produced by this gland (122,123,124,125). They also isolated 11-dehydrocorticosterone and 11-deoxycorticosterone as minor secretory products (121). In a later publication, Ward and Birmingham reported the identification of 18-hydroxy-11-deoxycorticosterone as the second most abundant steroid of the rat adrenal (126). Peron also identified this compound (127). In 1961 Peron reported, in addition, the isolation of 18-hydroxycorticosterone (128).

5b. Corticoid Secretion in the Mouse

The adrenal cortex of the adult mouse consists of an outer connective tissue capsule, of the zona glomerulosa and the zona fasciculata. The zona reticularis is, at best, poorly developed (43). However, if ACTH is injected into the animal for a period of ten days, the weight of the adrenal is increased, and a marked zona reticularis appears (43). This indicates that the mouse adrenal cortex is very responsive to ACTH.

It was not until 1956 that any interest was shown in the adrenocortical secretion of the mouse. At that time Hofmann (129) reported the presence of four ultraviolet absorbing areas on chromatograms obtained from extractions of incubated mouse adrenals. Two of these were tentatively identified as aldosterone and corticosterone. He reported, in one experiment only, the presence of a small amount of Porter-Silber positive material (4 μg per 100 mg adrenal). He concluded that the mouse does not usually produce Porter-Silber positive material. In 1956 Southcott et al. (130) reported the presence of 20α-dihydrocorticosterone and corticosterone in the plasma of normal and ACTH-treated mice. They obtained twice as much corticosterone as 20α-dihydrocorticosterone. ACTH increased the concentration of both compounds 3-fold. They concluded that 20α-dihydrocorticosterone is a tissue metabolite of corticosterone.

In 1958 Wilson et al. (32) reported minute amounts of Porter-Silber positive material in the serum of mice bearing ACTH-producing

tumors of the pituitary. They also described the presence of two C-19 compounds as well as a compound tentatively identified as corticosterone. The levels of corticosterone and of the Porter-Silber positive material were doubled in tumor-bearing animals. The level of the C-19 compounds was unaffected by ACTH. Bloch and Cohen (131) showed that, in vitro, corticosterone is the main steroid produced by normal mouse adrenal tissue, and that 11β-hydroxy- Δ^4-androstene-3,17-dione is a minor product. Corticosterone and 11β-hydroxy- Δ^4-androstene-3,17-dione were the major steroid hormones synthesized by adrenal tumor slices. Small amounts of other steroids were also produced. With successive transplantations of the tumor, responsiveness to ACTH was decreased with a subsequent decrease of corticosterone synthesis. The synthesis of 11β-hydroxy- Δ^4-androstene-3,17-dione was slightly increased.

Varon and Touchstone (132) reported recently that immature male mice are able to produce 17-hydroxylated steroids. They claim that adrenals from one month-old mice produce cortisol and corticosterone in equal amounts, as well as one other unidentified 17-hydroxycorticoid. Six month-old mice were reported by these authors to produce eight times as much corticosterone as cortisol. They interpreted these results by suggesting that androgens, in mature animals, inhibit the formation of 17-hydroxylated compounds. Contradictory evidence to the above interpretation was supplied by Huseby and Domingues (133). They reported that ovariectomized, young, female mice developed hyperplastic adrenals that were able to produce androgenically active steroids. Homogenates of these hyperplastic adrenals were shown to convert progesterone

to 17α-hydroxyprogesterone, indicating the presence of a 17-hydroxylase, usually absent in normal adrenals. Vinson and Jones (134) reported that adrenal glands of male and female mice are capable of converting progesterone to corticosterone and estrogens. The estrogens so formed are 16-oxoestrone and 17-epioestriol. Both are also produced in the mouse ovary.

Recently Raman et al. (135) showed that radioactive progesterone is converted, in vitro, by the mouse adrenal to 18-hydroxycorticosterone and aldosterone. In addition, three more radioactive zones were observed on Bush B_5 chromatograms.

In summary, it appears that the major steroids secreted by the vertebrates are cortisol and corticosterone. Among the mammals investigated so far, cortisol is usually the main steroid hormone produced. Exceptions are the rabbit, the rat, and the mouse, in which corticosterone predominates. Aldosterone represents a small per cent of the corticoid secretion of most terrestrial species. However, in the frog it is the major adrenal steroid. In this species it may have a major role in the retention of sodium by the skin.

(E) Purpose of this Study

At the time when this investigation was begun, little was known about the corticoid secretions of the mouse adrenal. However, it had been established, by several investigators, that corticosterone is

the major steroid hormone secreted by this species.

Previous work in this laboratory has provided detailed knowledge about the adrenal secretions of the rat. The purpose of this study was, therefore, to obtain more information concerning the identity of the steroids produced by the mouse adrenal, to further study the response to ACTH by adrenal glands *in vitro* and to compare the steroid hormones produced by the rat and the mouse, two closely related species.

II. METHODS AND MATERIALS

(A) Incubation

1. Preparation of Solutions Used

a. Incubation Medium (KRBG): Krebs-Ringer-bicarbonate solution containing 0.1M glucose (136). The solution was saturated with 95% O_2-5% CO_2.

b. ACTH-Containing Medium: A few milligrams of Nordic ACTH (20 international units (i.u.) per mg) were dissolved in 0.2-0.5 ml of 0.1N HCl and diluted with KRBG to give the required concentrations.

2. Incubation Procedure

Approximately 6 month-old male mice (Swiss, CF$_1$) weighing 25-30 gm were decapitated without anaesthesia. The adrenals glands were removed, cleaned free of fat and put intact into a Petri dish containing KRBG solution. The adrenals were then distributed, according to the design of the experiment, on a KRBG-moistened filter paper. This filter paper was kept in a Petri dish, which was covered to keep the atmosphere moist, and kept on ice. The adrenal tissue was weighed on a microtorsion balance and placed in 10 ml beakers; 2.0 ml of KRBG solution were added to beakers containing tissue, as well as to an equal number of control beakers containing no tissue. All beakers were incubated in a Dubnoff Metabolic Shaking Incubator under 95% O_2-5% CO_2 at 38°C for 30-60 minutes. At the end of this "pre-incubation" period,

the medium in each beaker was replaced by new ACTH-free or ACTH-containing medium. On certain occasions 100 ml-beakers were used. In that case the amount of medium added was 10 ml. The incubation was carried on for another 2 hours. At the end of this period, medium from each beaker was transferred to a test-tube and extracted once with an equal volume of methylene chloride. Steroid concentration was determined by ultraviolet measurements of the methylene chloride extracts. (This method will be described later).

For experiments in which the sole purpose was the collection of large amounts of steroids, 80 mice were used. The adrenals were not weighed but placed in 16 beakers so that each beaker contained 10 adrenals (about 25 mg of tissue). An equal number of control beakers containing no tissue was prepared. After pre-incubation, the medium was discarded; ACTH-free medium was added to 8 beakers, while ACTH-containing medium was added to the remaining 8 beakers (2.5 U per beaker, i.e. 10 U of ACTH per 100 mg of tissue). Incubation lasted for 8-10 hours, with change of medium every 2 hours. Media from all ACTH-containing beakers were pooled every 2 hours. The same procedure was followed with ACTH-free media. This pooled solution (16 ml) was extracted with an equal amount of methylene chloride. Media from control beakers containing no tissue were treated in identical fashion. The steroid concentration of the methylene chloride extracts was determined. The extracts were then evaporated to dryness for chromatography.

(B) Chromatography

1. Paper Chromatography

a. Preparation of Paper

Whatman No. 42 filter paper (47 cm long) was cut along the direction of the fibre to give sheets 18 cm wide consisting of strips 1 cm or 2 cm wide attached to a common head. These sheets were washed in a Soxhlet apparatus with methanol-benzene (1:1) for 2 days (137), hung in the dark to dry, and then stored in the dark until used.

b. Application of Steroids

Steroid-containing solutions and the corresponding blanks were evaporated to dryness under a stream of nitrogen and in a water-bath below 50°C. Methylene chloride, 0.2 ml, was added and the resulting solution was applied to the paper with a micro-pipette. The solution was confined to a small circle of about 0.5 cm in diameter. The solvent was evaporated with nitrogen during the application procedure. Reference steroids were included on each chromatogram.

c. Solvent Systems Used

(i) Toluene-Propylene Glycol (138)

Preparation of Strips for Spotting: Strips were wetted in a solution of 60 ml of methanol + 40 ml of propylene glycol, prior to the application of the steroids, and then blotted between two sheets of filter paper.

Solvents: 400 ml toluene + 100 ml propylene-glycol.

Temperature: Room temperature.

Equilibration time: 2-20 hours.

Running time: 6-10 hours or 30-72 hours.

When the chromatogram was developed over 10 hours, the effluent was sometimes collected in small beakers placed underneath each strip to be re-chromatographed.

(ii) Benzene-Propylene Glycol

Preparation of Strips for Spotting: as above.

Solvents: 400 ml benzene + 100 ml propylene-glycol.

Temperature: Room temperature.

Equilibration time: 2-20 hours.

Running time: 6-8 hours.

(iii) Cyclohexane-Benzene-Propylene Glycol

Preparation of Strips for Spotting: as above.

Solvents: 200 ml cyclohexane + 200 ml benzene + 100 ml propylene-glycol.

Temperature: Room temperature.

Equilibration time: 2-20 hours.

Running time: 6-8 hours.

(iv) Bush B_5 (139)

Stationary phase, 500 ml methanol + 500 ml water, was placed at the bottom of the chromatography tank. Strips were not wetted.

Solvents: 900 ml benzene + 100 ml ethyl acetate +
500 ml methanol + 500 ml water.
Temperature: Room temperature.
Equilibration time: 12-15 hours.
Running time: 4-6 hours.

Great care was taken to keep the temperature constant during the running time.

d. Drying and Elution of Chromatograms

After removal from the tanks, the chromatograms were hung in the dark overnight to dry. The appropriate ultraviolet-absorbing areas were located by scanning and comparison with the reference steroid. The paper in this region was cut into small pieces and placed into stoppered flasks. About 5 ml of methanol were added and the flasks were gently shaken in the Dubnoff incubator for 2-4 hours. At the end of the elution period, the methanol solutions were evaporated under nitrogen. For quantitative determinations an exact volume of methanol was added.

2. Thin-Layer Chromatography

a. Preparation of the Thin-Layer Plates

Selected microscope slides (2.5 by 7.5 cm) were used most of the time. When large amounts of material were to be chromatographed, larger glass plates (7.5 by 20 cm) were used.

After the required number of plates had been arranged on a

tray, or on a flat table, a wet cloth was passed over them. The coating mixture was prepared as follows: 22 gm of Silica gel G + a minute amount of "Luminiscent" (to cause the plates to fluoresce under ultra-violet light) + 48 ml of water. The mixture was shaken vigorously and applied, with a hollow glass roller, on the plates. Once the water has been added to the Silica, it is important to apply the mixture quickly. The plates were left overnight to dry and were then activated in an oven at 115°C for at least 1/2 hour.

b. Application of Steroids

Several samples were applied along the width of the plate. The same procedure for the application of the samples, as described above, was used. The small plates were developed in 200 ml beakers, with 8-10 ml of solvent placed at the bottom. Special glass jars were used for the larger plates. Specially fitting covers were placed on top of the glass jars; the beakers were covered with a Petri dish.

c. Solvent Systems Used

(i) Isopropyl alcohol-carbon tetrachloride (25:75).

(ii) Benzene-ethyl acetate (5:95).

(iii) Benzene-ethyl acetate-methanol (74:15:11).

The mixtures were shaken vigorously in the chromatogram jars; no further equilibration was required. At room temperature, the running time was 10-15 minutes for the small slides and 1-1.5 hours for the larger plates.

d. Elution from the Thin-Layer Plates

The silica gel containing ultraviolet-absorbing material was scraped into a test tube, and equal volumes of water and ethyl acetate were added. The steroids were extracted into the ethyl acetate layer (top layer) which was later evaporated.

3. Detection of Steroids on Paper

a. Ultraviolet Absorption

Paper chromatography strips were scanned on the Densicord (Photovolt Corp.). This procedure detects steroids possessing an α-β unsaturated ketone group.

b. Colour Reactions

Colour tests were performed on narrow strips (0.2 to 0.5 cm wide) cut out from developed chromatograms.

(i) Reduction of Tetrazolium

The paper strip was passed through a solution of the tetrazolium derivative M. and B. 1767, (2 mg of 2:5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride + 2 ml of 95% ethanol + 5 ml of 2.5N NaOH). It was then quickly passed through distilled water and blotted between two sheets of filter paper. Development of a purple colour is characteristic of a compound with a α-ketol group. The colour appears instantly.

(ii) Porter-Silber Reaction

This method measures compounds with a dihydroxyacetone side-chain (140). It also measures 21-hydroxysteroids with a 20 ---> 18 hemiketal linkage (with the exception of compounds containing, in addition, a hydroxyl group at C-11) (126). The paper strip was passed through the Porter-Silber reagent (140), and if the reaction was positive a yellow colour developed within 0.5-2 hours.

4. Detection of Steroids on Thin-Layer Plates

a. Ultraviolet Absorption

Ultraviolet (u.v.) absorbing areas on the thin-layer plates were detected by means of an u.v. lamp. The u.v.-absorbing spots stood out well against the fluorescing background.

b. Colour Reactions

(i) Charring

The thin-layer plates were sprayed uniformly with concentrated sulfuric acid and heated on a hot plate for 40-60 seconds. The location of a compound was indicated by a dark spot. This is a test for any carbon-containing compound.

(ii) Reduction of Tetrazolium

The thin-layer plates were sprayed with the M. and B. reagent. A purple colour appeared immediately when an α-ketol-containing compound was present.

(iii) Porter-Silber Reaction

The Porter-Silber reagent was sprayed on the thin-layer slide. A yellow colour appeared if a compound with the dihydroxyacetone side-chain was present. Appearance of the yellow colour took 5-20 minutes.

(C) Tests on Eluted Steroids

1. Absorption Spectra

All spectrophotometric measurements, excepting the infrared spectra, were obtained in the Beckman DK-2 recording spectrophotometer. The samples were read against an appropriate blank in the reference microcuvette. The infrared spectra were determined in a Perkin-Elmer single beam spectrometer, model 12C, with sodium chloride optics.

a. Ultraviolet Absorption Spectra

A solution of the steroid in methanol or methylene chloride was read against its blank solution. The wavelength range was 215-280 mμ for methanol and 230-280 mμ for methylene chloride. The amount of steroid present was calculated by comparing the difference between the optical densities at 240 mμ and 260 mμ with a similar calculated density of a known amount of a standard solution of cortisol. The α-β unsaturated ketones yielded u.v. spectra with a maximum between 238-242 mμ.

b. Infrared Spectra

Crystals or an aqueous solution of a steroid were mixed with about 40 mg of potassium bromide. A transparent disc was prepared by compressing the mixture for 1 minute at 8000-9000 lbs. in a Calver press.

c. Sulfuric Acid Spectra

A solution of steroid in concentrated sulfuric acid, 15 μg in 1.0 ml, was allowed to stand at room temperature for two hours. The absorption spectrum was then determined over the wavelength range 200-600 mμ (141).

2. Colour Tests for Functional Groups

a. Reduction of Tetrazolium

The method as described by Birmingham and Kurlents (62) was used. To a solution containing 0.5-2 μg of steroid in 0.05 ml of ethanol were added 0.05 ml of an 0.15% ethanolic solution of 2:5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride and 0.05 ml of a solution containing 0.3 ml of a 10% solution of tetramethylammonium hydroxide diluted to 10 ml with ethanol. After incubation at 28°C in the dark for 20 minutes the solution was cooled in ice water, neutralized with 0.05 ml of 0.045M acetic acid in ethanol. The spectra were determined over the wavelength range 400-700 mμ. All α-ketol containing compounds had spectra with a maximum at 510 mμ. If more than 2 μg of steroids were present in solution, then correspondingly larger volumes of reagent were used.

b. Porter-Silber Reaction

The method, as described by Porter and Silber (140) was used. To a dry steroid residue (1-3 μg), in a test tube, 0.5 ml of the phenylhydrazine reagent was added. The reagent consists of: 65 mg of recrystallized phenylhydrazine hydrochloride dissolved in a solution composed of 62 ml of concentrated sulfuric acid, 38 ml of water and 50 ml of ethanol. The steroid solutions were kept in the dark for 16-24 hours. The spectra were determined over a range of 380-500 mμ. Compounds with a dihydroxyacetone side-chain yield spectra with a maximum at 410 mμ.

(D) Formation of Derivatives

1. Acetylation

The procedure of Zaffaroni et al. (142) was used for the acetylation of corticosterone. To 10-100 μg of dry steroid were added 3 drops of acetic anhydride and 5 drops of pyridine. The solution was mixed and left overnight in the dark. The solvents were then evaporated under a stream of nitrogen and the acetylated product was chromatographed.

1a. Acetylation of Aldosterone

The method of Mattox et al. was used (143). To a solution of 80 μg in 1 ml of glacial acetic acid were added 1.2 ml of a mixture consisting of 1 ml of acetic anhydride and 5 ml of 3M pyridine in glacial acetic acid. After 75 minutes at room temperature, 10 ml of chloroform were added and the mixture cooled in ice, then 3 ml of 1N hydrochloric acid were added. The mixture was shaken vigorously to allow

the chloroform to extract the steroid. The chloroform layer was removed and evaporated to dryness for chromatography.

2. Oxidation of Aldosterone 21-Acetate to 11,18-Lactone 21-Acetate (143)

A solution of aldosterone 21-acetate in 1 drop of acetic acid was cooled to 13°C. A solution consisting of 1 gm of chromic acid in 1 ml of water and 99 ml of glacial acetic acid was also cooled to 13°C, and 1 drop of this solution was added to the aldosterone-acetate solution. After 10 minutes at this temperature, 0.5 ml of water and 5 ml of ethyl acetate were added. The steroid was extracted with ethyl acetate, which was then evaporated to dryness and prepared for chromatography.

III. RESULTS

(A) Factors Affecting Steroid Production by Incubated Mouse Adrenals

1. Endogenous Steroid Production

Endogenous production refers to the steroid output by adrenals incubated in the absence of ACTH, after a pre-incubation period of 30 minutes to 1 hour. Methylene chloride extracts of incubation media yielded u.v. absorption spectra with a maximum at 238-240 mμ, characteristic of an α-β unsaturated ketone.

Table I shows the amount of u.v.-absorbing material obtained when mouse adrenals are incubated in KRBG, with replacement of medium every two hours. The amount of steroids produced every two hours was determined over a period of ten hours. The steroid production varied between 11-14.5 μg/100 mg adr. tissue/2 hrs. This variation was even smaller when the steroid production was calculated per unit gland, rather than per unit gland weight (14.8-15.8 μg/40 adrenals/2 hrs).

2. Effect of Varying ACTH Concentrations

Increasing the amount of ACTH added to the incubating medium increased the amount of steroids produced (Figs. 3 and 4). The dose-response curve was linear between 100-1000 mU/100 mg adrenal weight (Fig. 3), and logarithmic at concentrations above 1000 mU (Fig. 4). There was no fall-off in the log-dose-response curve, even at the highest concentration of ACTH that could be kept in solution. The amounts of

TABLE I

STEROID PRODUCTION BY MOUSE ADRENALS INCUBATED IN KREBG

Duration of Incubation	Average Production (µg/100 mg adr. wt.)/2 hr. + S.E.	Average Production µg/40 adrenals*/2 hr. + S.E.
1st 2 hrs.	11.0 ± 1.95 (12)	15.8 ± 1.83 (5)
2nd 2 hrs.	14.0 ± .28 (3)	15.3 ± 1.67 (6)
3rd 2 hrs.	12.5 ± 1.64 (3)	14.8 ± 1.31 (6)
4th 2 hrs.	14.6 ± 2.7 (3)	15.6 ± 1.52 (4)
5th 2 hrs.	-	15.6 ± 3.38 (4)

Figures in parentheses represent number of experiments.

* about 100 mg.

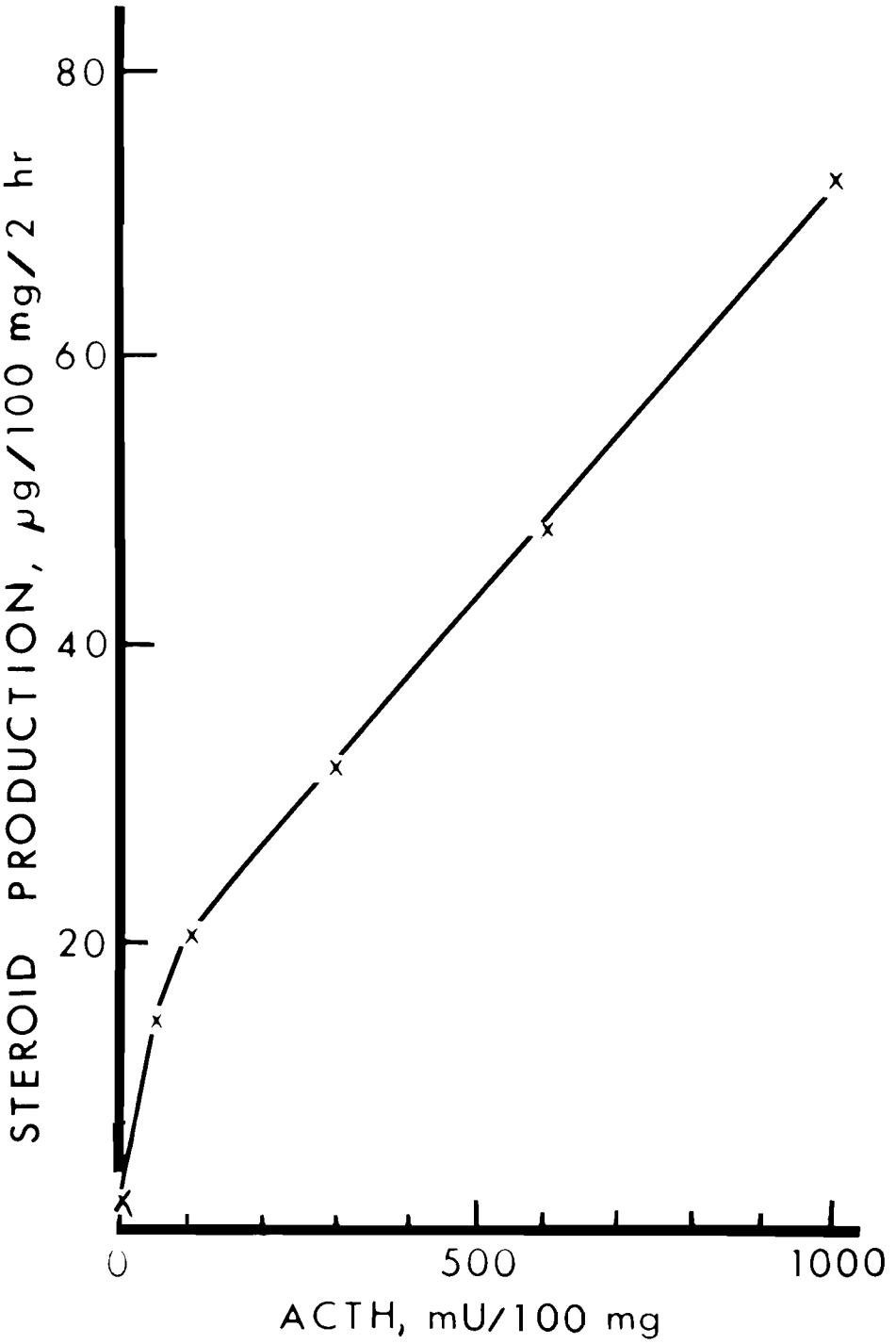


FIG. 3. Dose-response curve obtained with incubated mouse adrenals. Output of total ultraviolet-absorbing lipids plotted against amount of ACTH added per 100 mg tissue. Each point is based on the output of 6 intact adrenals.

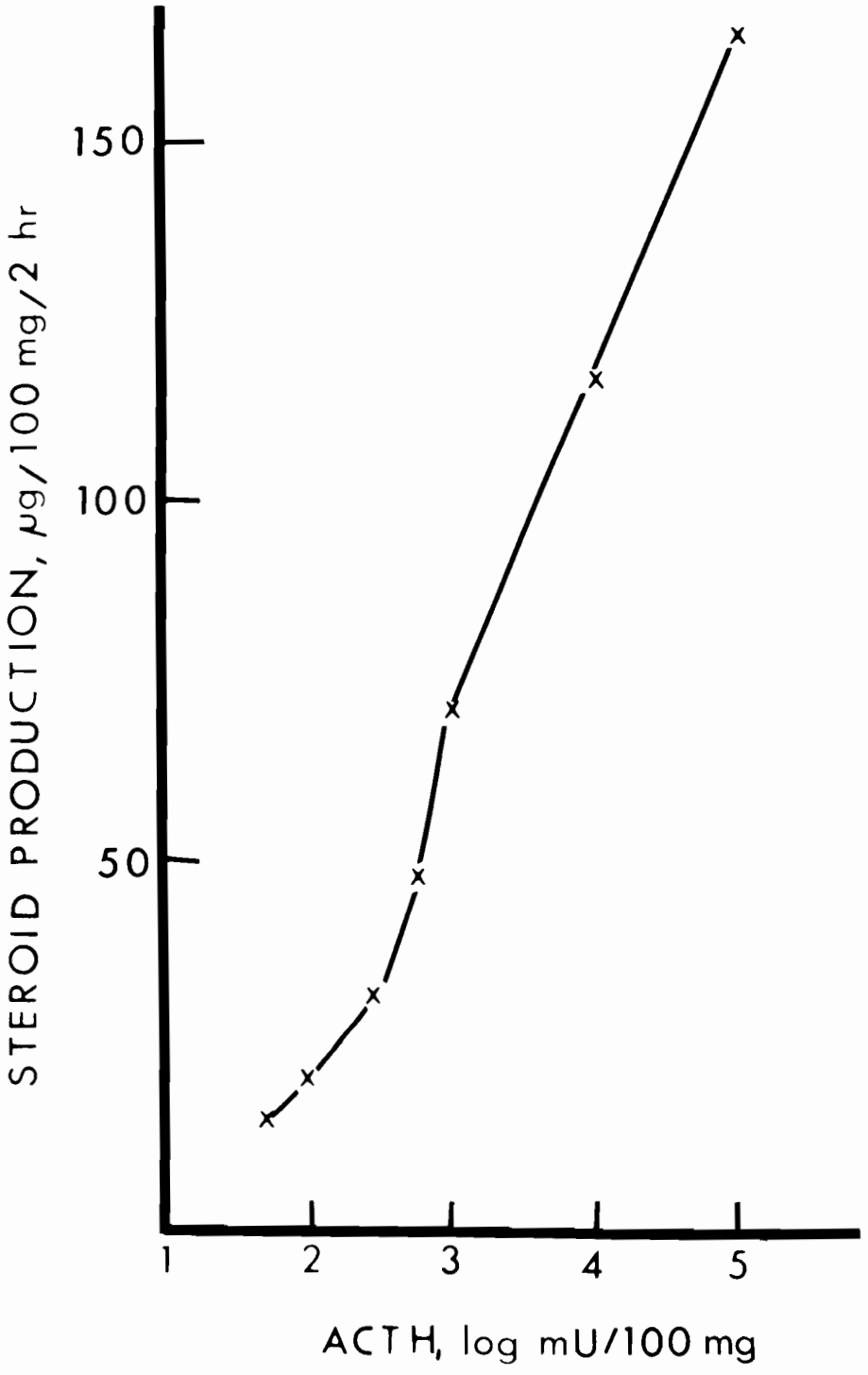


FIG. 4. Dose-response curve obtained with incubated mouse adrenals. Output of total ultraviolet-absorbing lipids plotted against the logarithm of the dose. Each point is based on the output of 6 intact adrenals.

steroids produced in this experiment varied from 2.6 µg/100 mg adr. wt./ 2 hrs in the absence of ACTH to 164 µg/100 mg adr. wt. at the highest dose tested (100,000 mU of ACTH/100 mg adr. wt.).

3. Effect of Time on the Response to ACTH

The production of steroids, in the absence of added ACTH, was remarkably unaffected by time, even for as long an incubation period as ten hours. The steroid production in response to ACTH, however, decreased sharply after four hours of incubation (Fig. 5). The ratio of stimulated to unstimulated production ranged from an average of about 7 during the first four hours to 1.6 near the end of the ten-hour incubation (Table II). The average overall increase in the steroid production due to ACTH, over the entire ten-hour period, was 380%.

4. Effect of Calcium on the Steroid Production

In two experiments, mouse adrenals were preincubated for 45 minutes in a calcium-free medium. At the end of this period, one group of adrenals was incubated in a KRBG-ACTH solution, while the other group - adrenals from the same animals - was incubated in a corresponding medium lacking calcium. The incubation was carried out for 4 hours, with change of medium every 2 hours.

Table III compares the steroid production of adrenals incubated in a KRBG-ACTH solution with the steroid production of adrenals incubated in calcium-free medium. ACTH-stimulated adrenals, incubated without calcium, produced on the average 2 1/2 times less steroid than adrenals

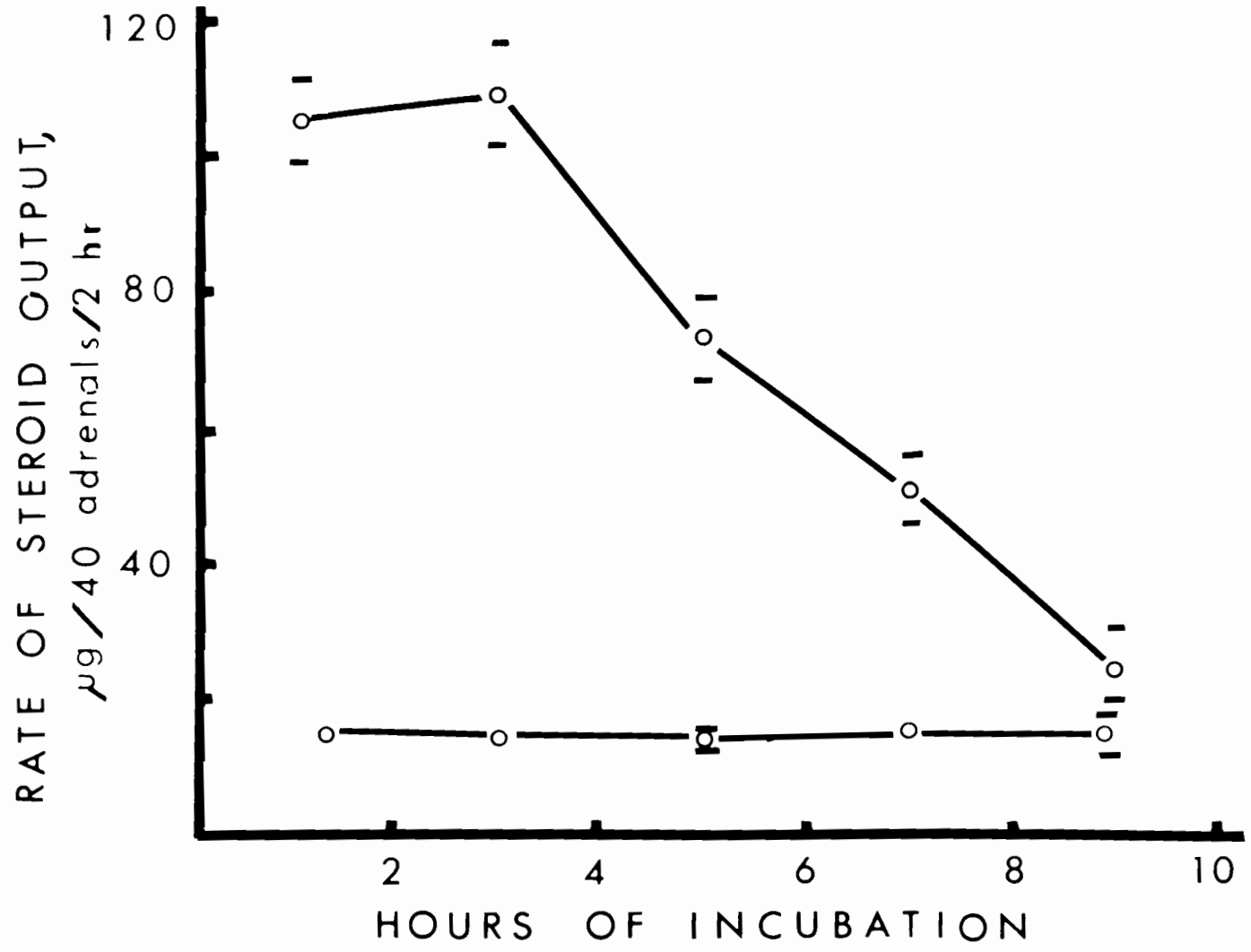


FIG. 5. Effect of time on the output of ultraviolet-absorbing lipids by incubated mouse adrenals. Top curve, ACTH-stimulated steroid production: lower curve, unstimulated production. Average values for 4-9 experiments are shown; bars indicate standard errors where they exceed the size of the symbol.

TABLE II

STEROID PRODUCTION BY MOUSE ADRENALS INCUBATED WITH AND WITHOUT ACTH

Duration of Incubation	Average Production µg/40 adrenals/2 hr. + S.E.		Ratios of stimulated to unstimulated production by the same animals
	No ACTH	ACTH*	
1st 2 hrs.	15.6 ± 1.51 (7)	105.7 ± 7.0 (8)	6.8
2nd 2 hrs.	14.6 ± 1.39 (8)	109.7 ± 8.0 (9)	7.5
3rd 2 hrs.	14.5 ± 1.90 (8)	73.9 ± 5.2 (8)	5.1
4th 2 hrs.	16.0 ± 1.35 (6)	52.8 ± 4.8 (9)	3.3
5th 2 hrs.	15.6 ± 3.38 (4)	25.4 ± 6.2 (5)	1.6
TOTAL	76.2	367.6	4.8

Figures in parentheses represent number of experiments.

* About 10 U/100 mg adrenal tissue.

TABLE III

EFFECT OF CALCIUM ON THE STEROID PRODUCTION BY INCUBATED MOUSE ADRENALS

Experiment	ACTH (mU/100 mg adr.)	Incubation Period	Steroid Production (µg/100 mg adr. wt./2 hrs) with Ca	without Ca	Ratio of Ca to no Ca
a, b	300	1st 2 hrs	43 ± 14	14 ± 0	3.1
	300	2nd 2 hrs	91 ± 12	34 ± 10	2.7
c, d	600	1st 2 hrs	53 ± 11	20 ± 4	2.6
	600	2nd 2 hrs	92 ± 4	47 ± 31	1.9
e	None	1st 2 hrs	-	1 ± 1	-
	None	2nd 2 hrs	-	4 ± 4	-

The average output of ultraviolet-absorbing steroids from two incubated samples is shown ± range.

incubated in complete medium. During the second incubation period, with fresh ACTH and medium, the steroid production was considerably greater than during the first incubation period. Doubling the dose of ACTH did not produce a significant increase in the steroid output. Minute amounts of steroids were produced in the absence of both calcium and ACTH.

5. Effect of Volume of Medium on Steroid Production

Several experiments were done in order to determine whether the volume of the incubation medium is an important factor in steroid production in the presence of any given amount of ACTH. Mouse adrenals from the same animals were divided into two equal groups. Adrenals from each group were distributed among beakers containing varying amounts of ACTH. One group of adrenals was incubated in 2 ml of medium, while the other group, from the same animals, was incubated in 10 ml of medium.

From Fig. 6 it is evident that the dose-response curve for adrenals incubated in 2 ml of medium does not vary significantly from the dose-response curve for adrenals incubated in 10 ml. The range tested was 50 to 100,000 mU/100 mg adr. wt.

6. Steroid Production by Mouse and Rat Adrenal

Table IV shows the amount of steroids produced, in three experiments, by ACTH-stimulated rat and mouse adrenals. Since one rat adrenal weighs about 6 times as much as a mouse adrenal, in experiments

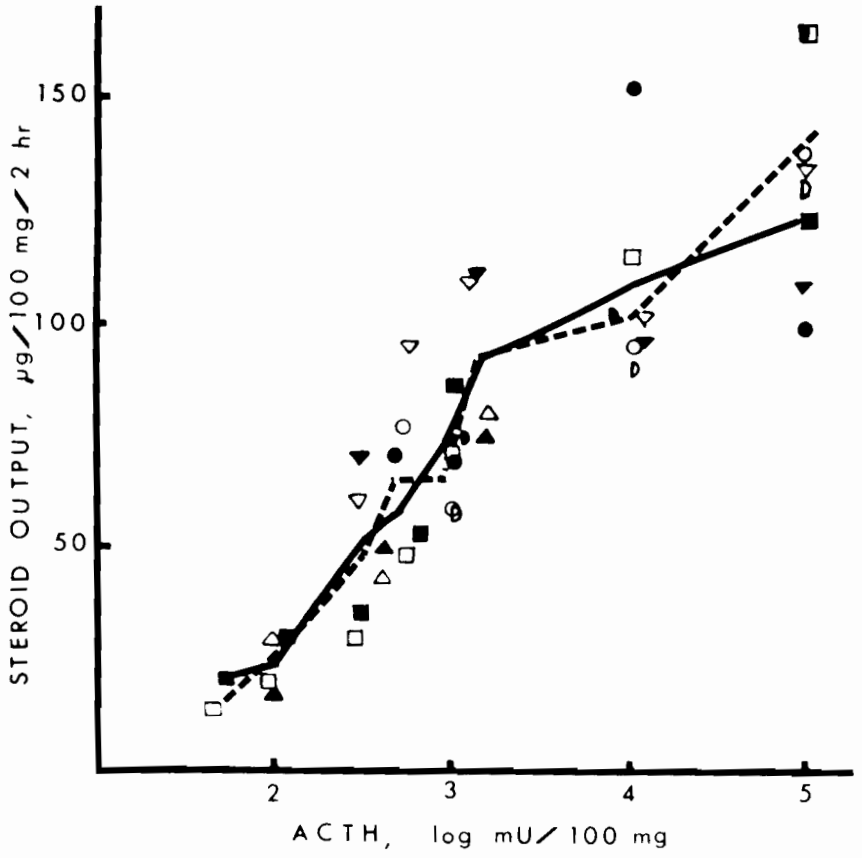


FIG. 6. Response by 6-8 intact mouse adrenals from the same animals to a given amount of ACTH, dissolved in 2 ml (solid symbols and line), and in 10 ml of medium (open symbols and broken line). The lines were drawn through the arithmetic averages obtained in different experiments with the same dose.

TABLE IV

COMPARISON OF THE STEROID PRODUCTION BY MOUSE AND RAT ADRENALS IN RESPONSE TO ACTH

ACTH Concentration (mU/100 mg adr. wt.)	Steroid Production (µg/100 mg of adr. wt./2 hr.)								
	EXP. 1			EXP. 2			EXP. 3		
	Rat	Mouse	Ratio	Rat	Mouse	Ratio	Rat	Mouse	Ratio
0	12.2	33.2	2.7	16.1	17.4	1.1	-	-	-
250	-	-	-	37.5	65.5	1.7	49.3	70.1	1.4
500	28.2	74.0	2.6	45.7	95.0	2.1	-	-	-
1000	39.9	68.4	1.7	49.0	107.5	2.2	-	-	-
10,000	69.0	124	1.8	67.5	100.3	1.5	-	-	-
100,000	64.4	118.8	1.8	76.3	121.5	1.6	-	-	-

Each value in Experiments 1 and 2 is an average of two.

The values in experiment 3 are an average of 4.

1 and 2 one intact rat adrenal and 6 intact mouse adrenals were incubated in separate beakers containing the same amount of ACTH per 100 mg adrenal weight. The steroid production per 100 mg adrenal weight was calculated. In experiment 3 the same procedure was followed, but in order to have the same number of rat adrenals as mouse adrenals, 6 rat adrenals were used. The steroid production per 100 mg adrenal weight was determined. The ratio of the amount of steroids produced, in response to ACTH, by mouse adrenals to the amount produced by rat adrenals varies from a low of 1.4 to a high of 2.6. However, in all cases it is evident that the mouse adrenal is more responsive to ACTH than the rat adrenal. These results are also shown in Fig. 7.

The results would have been similar if sectioned adrenals had been compared, as there was no marked difference between the steroid output of halved and intact rat adrenals at different doses of ACTH, at least up to 4000 mU (Fig. 8). This also applied to mice; halved mouse adrenals incubated with 200 mU of ACTH per 100 mg produced 78 µg of u.v.-absorbing lipids per 100 mg adrenal weight per 2 hours, compared to 70 µg obtained with intact glands from the same animals.

7. Summary of Part A

1. The dose-response curve to ACTH appears linear between 100-1000 mU of ACTH/100 mg adrenal weight and logarithmic between 1000-100,000 mU of ACTH.

2. There was little or no levelling-off in the log-dose-response curve at the highest concentrations of ACTH that could be kept

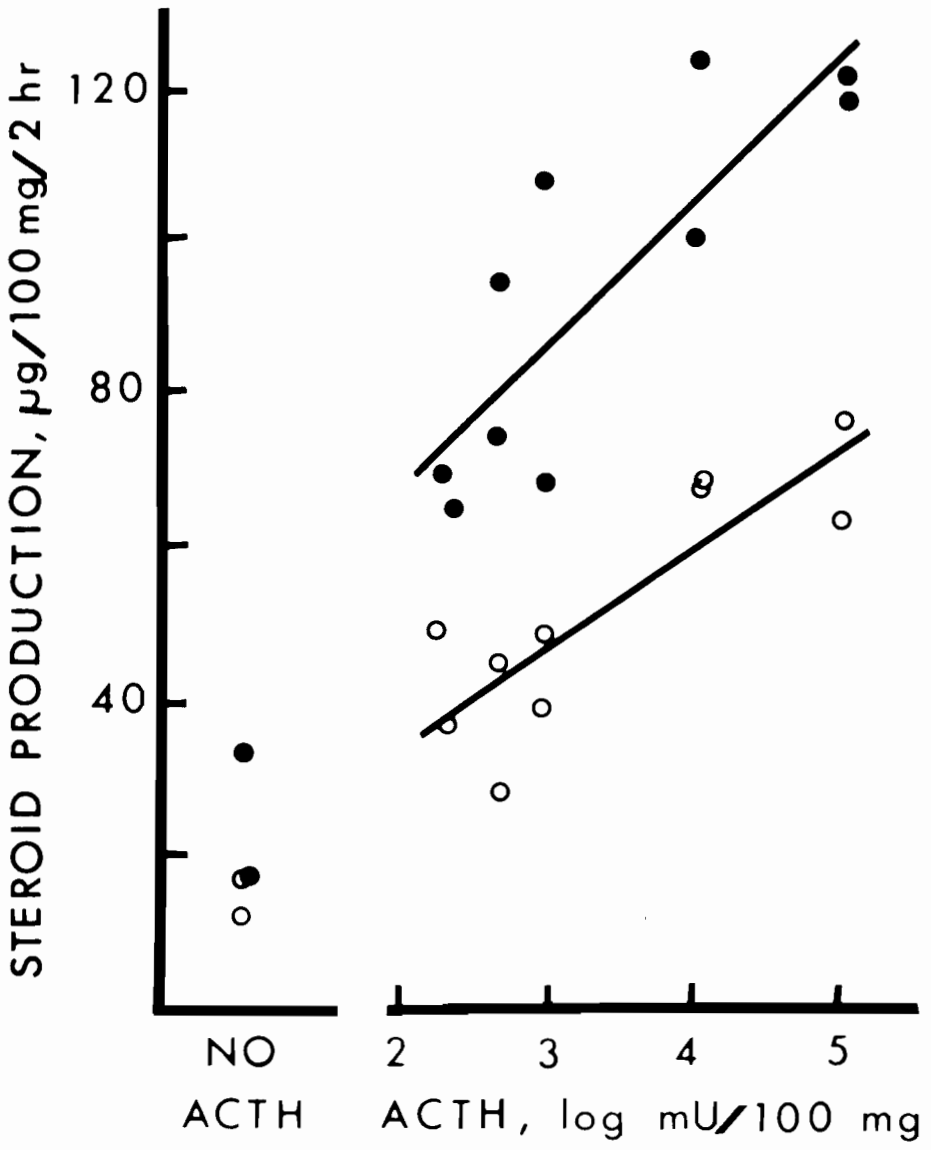


FIG. 7. Comparison of the production of total ultraviolet-absorbing lipids by intact mouse (solid symbols) and rat (open symbols) adrenals. Each point was obtained with 6 mouse adrenals or 1 rat adrenal. Regression lines are shown ($r > 0.8$ in either case; the difference between the two slopes of the regression lines was not significant).

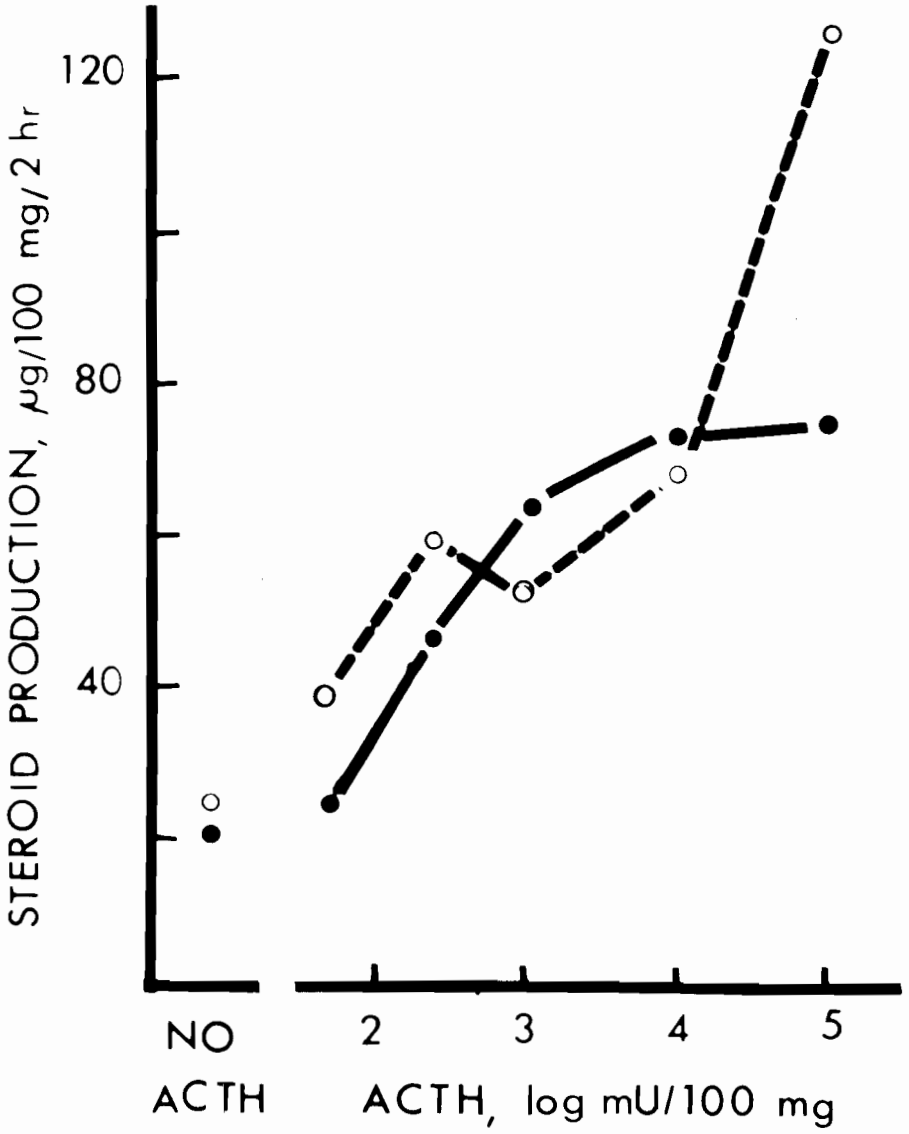


FIG. 8. Comparison of the production of total ultraviolet-absorbing lipids by intact (solid symbols) and quartered (open symbols) rat adrenals from the same animal. Each point was obtained with one adrenal.

in solution.

3. Over a 10-hour period the endogenous steroid production remained constant. The ACTH-stimulated production remained constant for the first 4 hours, and then fell rapidly with time. The average overall increase in the steroid production due to ACTH, over the entire 10-hour period, was 380%.

4. The presence of calcium is essential in the incubation medium for optimal stimulation by ACTH.

5. There was no difference in the ACTH-stimulated steroid production by adrenals incubated in 2 and 10 ml of medium.

6. Mouse adrenals produce more steroids than rat adrenals when incubated with the same dose of ACTH.

(B) General Characteristics of the Steroids Produced by Incubated Mouse Adrenals

1. Major Steroid Fractions Produced by Incubated Mouse Adrenals

Methylene chloride extracts of media from mouse adrenals incubated with or without ACTH yielded, on chromatography in toluene-propylene glycol (TPG) for 72 hours, three major fractions. These shall be designated in order of decreasing polarity, fractions M_1, M_2 and M_3. The mobilities of these fractions, relative to standard corticosterone (R_B), were 0.15, 0.40 and 1.0 respectively (Fig. 9, lower part). For reference purposes standard cortisol, cortisone, 11-deoxycortisol and

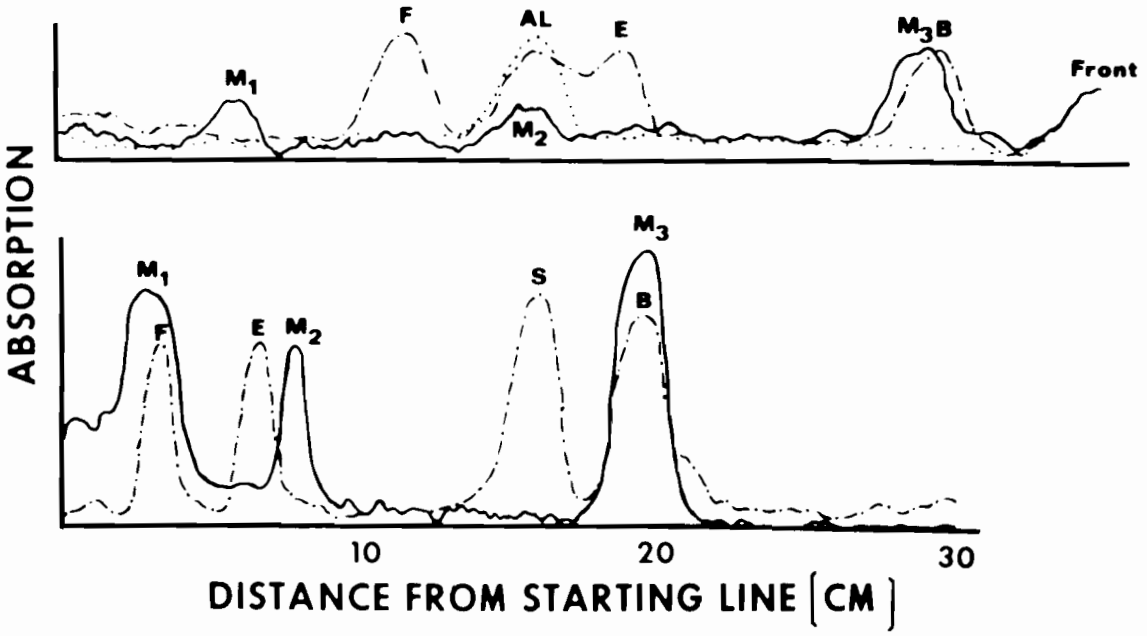


FIG. 9. Paper scans of ultraviolet-absorbing areas obtained from extracts of mouse adrenal incubation media chromatographed in toluene-propylene glycol (lower figure) and in the Bush B_5 system (upper figure). Broken lines - chromatographed standards, cortisol (F), cortisone (E), aldosterone (Al), 11-deoxycortisol (S), and corticosterone (B).

corticosterone were mixed and chromatographed. Their mobilities relative to standard corticosterone were found to be 0.18, 0.34 and 0.81 respectively. Fig. 9 shows that adrenal fraction M_1 is slightly more polar than standard cortisol (compound F), adrenal fraction M_2 is less polar than standard cortisone (compound E), while fraction M_3 runs with the same mobility as standard corticosterone. There was no evidence of a fraction that would correspond to 18-hydroxy-11-deoxycorticosterone (18-OH-DOC), found in the rat.

2. Ultraviolet Absorption Spectra of Eluted Steroid Fractions

All regions from chromatograms of adrenal extracts were eluted and the u.v. absorption spectrum of each region determined over a range of 280-220 mμ. Eluates of every zone yielded u.v. absorption spectra with an absorption maximum at 238-240 mμ, characteristic of an α-β unsaturated ketone (Fig. 10).

3. Effect of ACTH on the Production of Each Steroid Fraction

Paper chromatograms of adrenal incubation extracts were scanned on the Densicord, and each of the u.v.-absorbing zones was eluted. Table V shows the average amounts eluted from each zone. It demonstrates that fraction M_3 is produced in far greater amounts in response to ACTH than the two more polar fractions. A comparison of the ACTH-stimulated production with the endogenous production, over the entire 10-hour period, shows a 2.8-fold increase in the production of the most polar material, a 1.9-fold increase in the production of fraction M_2 and a 8.3-fold increase in the lipid with the mobility of corticosterone.

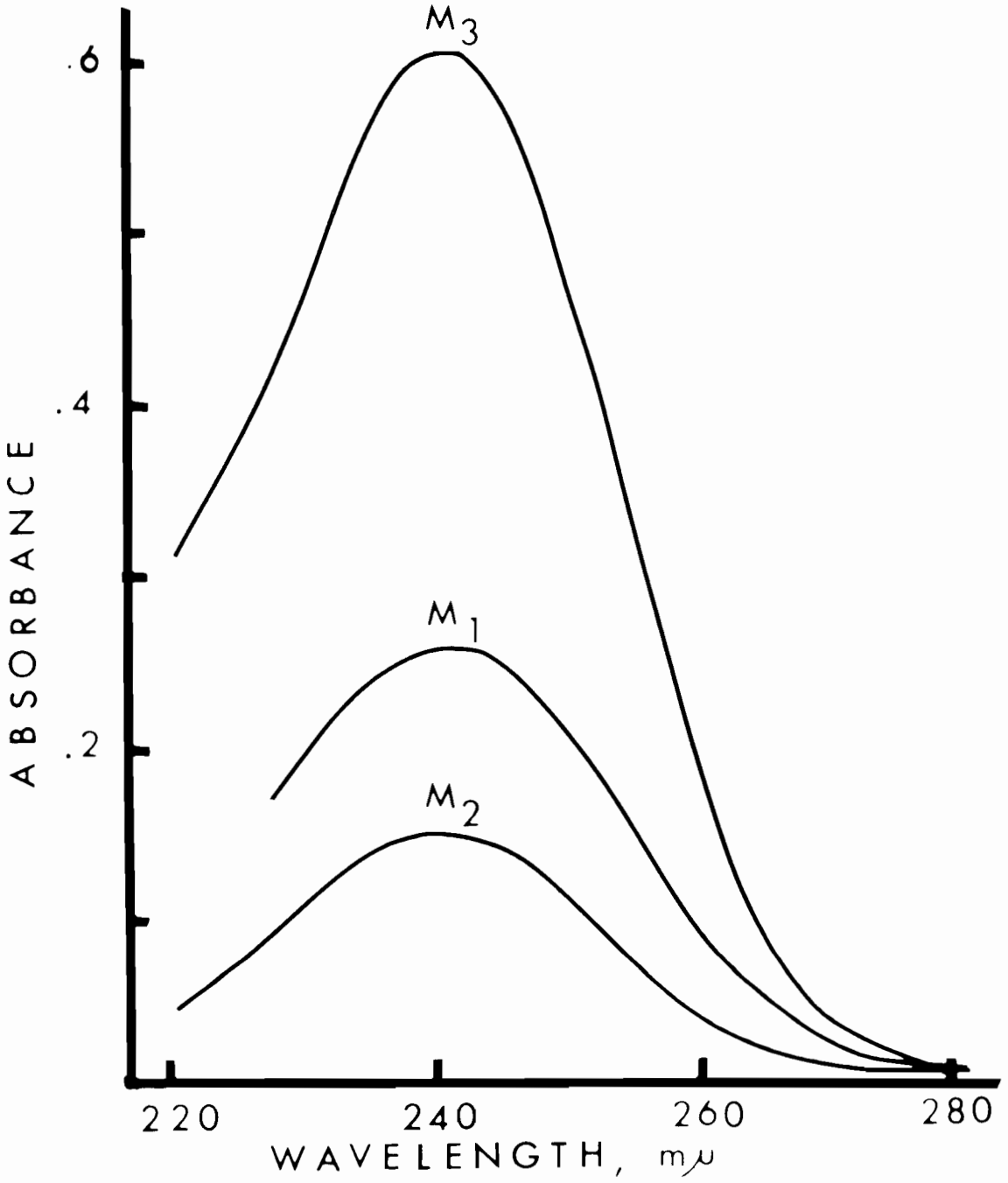


FIG. 10. Ultraviolet-absorption spectra of mouse adrenal lipid fractions in methanol, eluted from toluene-propylene glycol chromatograms.

TABLE V

COMPARISON OF THE PRODUCTION OF THE 3 MAJOR STEROID FRACTIONS
BY MOUSE ADRENALS INCUBATED WITH AND WITHOUT ACTH

	Average Recovery (μg/40 adrenals/10 hrs)			Per Cent of Total Eluate		
	Fraction M_1	Fraction M_2	Fraction M_3	Fraction M_1	Fraction M_2	Fraction M_3
+ ACTH*	32.1	22.2	204	12.4	8.6	79
- ACTH	11.5	11.5	25	24.0	24.0	52.0
Ratio of +ACTH to -ACTH	2.8	1.9	8.3			

* About 10,000 mU per 100 mg adrenal weight.

In the presence of ACTH, fraction M_3 represented 79% of the eluted material, fractions M_1 and M_2 12.4 and 8.6% respectively. In the absence of ACTH, the percentages were 52% for fraction M_3, and 24% each for fraction M_1 and M_2. It will be noted that fraction M_1 contained significantly more lipid than fraction M_2 in the presence of ACTH, but not in its absence.

4. Effect of Time on the ACTH-Stimulated Production of Each Steroid Fraction

Table VI shows the amounts of each steroid fraction produced per 2 hrs over a 10-hr incubation period. The fall-off with time in the production of the steroid fractions is best demonstrated when the amount of each fraction eluted per 2 hours is calculated as per cent of the total eluted fraction.

Each steroid fraction is produced at a nearly constant rate for the first 4 hours of incubation. The amount of each fraction produced during that period is about 60% of the total, the remaining 40% is produced during the last 6 hours of incubation (Table VI). Thus with prolonged incubation the production rate, in response to ACTH, decreases appreciably. This is similar to the curve of ACTH-stimulated production vs. time for total steroids (Fig. 5). The fall-off in production seems to be the same for all 3 fractions. The output of fraction M_2 is significantly increased by ACTH only during the first four hours.

TABLE VI

DISTRIBUTION, WITH TIME, OF STEROID FRACTIONS PRODUCED BY INCUBATED MOUSE ADRENALS

Incubation Period (2 hrs each)	Average Recovery µg/40 adr./2 hrs + S.E.			Per Cent of Total Fraction Produced in 10 Hrs		
	Fraction M_1	Fraction M_2	Fraction M_3	Fraction M_1	Fraction M_2	Fraction M_3
ACTH present						
1st (N=4)	8.5±1.7^{+}	7.4±1.0^{++}	45.2±3.4^{+++}	26.5	33.4	22.2
M_1-M_2	1.1±0.9					
2nd (N=5)	9.5±1.7^{++}	6.2±1.0^{++}	58.7±9.7^{+++}	29.6	28.0	28.8
M_1-M_2	3.3±0.6**					
3rd (N=4)	5.4±0.8^{+}	3.4±0.7	39.8±4^{+++}	16.8	15.3	19.5
M_1-M_2	2.0±0.2**					
4th (N=3)	4.9±0.2^{++}	2.8±0.3	38.8±10.8^{+}	15.3	12.6	19.0
M_1-M_2	2.1±0.3*					
5th (N=2)	3.8±0.6	2.4±0.4	21.8±8.3^{+}	11.8	10.7	10.5
M_1-M_2	1.4			100.0	100.0	100.0
ACTH absent						
10 hrs; per 2 hours (N=4)	2.3±0.6	2.3±0.5	5.0±0.5			
M_1-M_2	0.0±0.2					

$^{+}$ significantly different from value obtained in the absence of ACTH, $P < 0.05$, $^{++} < 0.01$, $^{+++} < 0.001$ (group comparisons);

* significant difference between M_1 and M_2, $P < 0.05$, ** < 0.01.

5. Summary of Part B

1. The mouse adrenal produces, in vitro, 3 major steroids, which run in TPG with mobilities similar to standard cortisol, cortisone, and corticosterone.

2. Each of these 3 steroids absorbs u.v. light, with a maximum at 238-240 mμ, indicating the presence of an α-β unsaturated ketone.

3. The steroid with the mobility of standard corticosterone represents about 80% of the total steroids eluted from the 3 major zones. The other 2 steroids make up the remaining 20%.

4. Fraction M_3 responds best to ACTH. (There is an 8.3-fold increase in production of this fraction after ACTH stimulation, but only a 1.9-2.8-fold increase in the other two fractions.)

5. Sixty per cent of each fraction is produced during the first 4 hours of incubation with ACTH; after that there is a fall-off in the production. The decrease in the rate of adrenal output is about the same for all three steroid fractions.

(C) Identification of the Steroid Fractions Produced by Incubated Mouse Adrenals

1. Ultraviolet Absorption, Reducing Power and Porter-Silber Reaction

Eluates from chromatograms of adrenal incubation media were tested for their ability to absorb ultraviolet light, with a λ_{max} at 240

mμ, to reduce the tetrazolium derivative M. and B. 1767, and to react with the Porter-Silber reagent.

As mentioned previously, absorption of u.v. light with a maximum at 240 mμ indicates the presence of an α-β unsaturated ketone. The M. and B. reaction is specific for compounds possessing an α-ketol group, while the Porter-Silber reaction measures compounds with a dihydroxyacetone side-chain, or a 21-hydrogenated steroid containing a 20 ---> 18 hemiketal linkage (excepting steroids containing, in addition, a hydroxyl group at C-11).

Table VII shows the results of these tests performed on the eluates of each steroid fraction. In addition, spot tests were also applied to these compounds on chromatography strips, and they confirmed the results obtained with eluates.

From these results, it may be concluded that each fraction possesses an α-β unsaturated ketone (since all absorb u.v. light near 240 mμ), that an α-ketol group is common to all of them, with the possible exception of fraction M_1. Since all fractions reacted negatively with the Porter-Silber reagent, the presence of hydrocortisone and cortisone may be excluded from all of them. The latter conclusion was further supported by the sulfuric acid spectra of fractions M_1 and M_2. Both gave spectra with a maximum near 290 mμ, characteristic of an α-β unsaturated ketone, but no maxima at longer wavelengths were observed, as are found in hydrocortisone and cortisone. As expected, of the reference steroids only hydrocortisone and cortisone gave a positive Porter-Silber reaction.

TABLE VII

LIST OF SPECIFIC PROPERTIES OF EACH STEROID FRACTION SEPARATED BY CHROMATOGRAPHY

	Tests done on eluates from chromatograms			Spot tests on chromatogram paper strips		
Compound	Reducing	Porter-Silber	λ_{max} 240 mμ U.V. absorption	Reducing	Porter-Silber	λ_{max} near 240 mμ U.V. absorption
Fraction M_1	+ (faint)	-	+	+ (faint)	-	+
Fraction M_2	+	-	+	+	-	+
Fraction M_3	+	-	+	+	-	+
Fraction M_4	Not done	Not done	Not done	+*	-	+
Hydrocortisone	+	+	+	+	+	+
Cortisone	+	+	+	+	+	+
Aldosterone	+	-	+	+	-	+
Corticosterone	+	-	+	+	-	+

* Component with the mobility of 11-dehydrocorticosterone.

2. Fraction M_1

a. Chromatographic Mobilities

Table VIII compares the chromatographic mobilities of fraction M_1 with that of standard hydrocortisone in three different systems of chromatography. In toluene-propylene glycol (TPG), fraction M_1 was slightly more polar than hydrocortisone, however in the Bush B_5 system it moved with one-half the mobility of hydrocortisone (Fig. 9).

After the chromatogram was allowed to develop in TPG, fraction M_1 was eluted and subsequently chromatographed on a thin-layer plate in 5% benzene-95% ethyl acetate (Table VIII). In that system fraction M_1 separated into three components of mobilities with respect to hydrocortisone (R_F) of .20, .40, 1.0 respectively. The major component, with R_F .40 moved with standard 18-hydroxycorticosterone. However, in contrast to the chromatographed standard, which did not reduce tetrazolium, it gave a faint positive spot test with tetrazolium on the thin-layer plate, as did the most polar component with R_F .20. The third component did not reduce tetrazolium.

b. Evidence that Fraction M_1 is not Hydrocortisone

Contrary to what has been reported in the literature, the following evidence strongly suggests that fraction M_1 is not hydrocortisone.

(i) Fraction M_1 always failed to react with the Porter-Silber reagent, which indicates lack of the dihydroxyacetone side-chain present in hydrocortisone.

TABLE VIII

MOBILITIES OF FRACTION M_1 AND OF SOME REFERENCE STEROIDS

Compound	Paper Chromatography			Thin Layer Chromatography					
	TPG	Bush B_5		5% Benzene in Ethyl Acetate					
	R_B	R_B	R_f	R_F			R_f		
				C_1	C_2	C_3	C_1	C_2	C_3
Fraction M_1	.15	.20	.17	.2(+)	.4(+)	1.0(-)	.06(+)	.12(+)	.29(-)
Hydrocortisone	.18	.39	.33					.29(+)	
18-OH Corticosterone	-	-	-		.40(-)			.12(-)	

Reaction with tetrazolium indicated in brackets.

TPG - Toluene-propylene glycol system.
R_B - Mobility with respect to corticosterone.
R_F - " " " " hydrocortisone.
R_f - " " " " the solvent front.

In benzene-ethyl acetate, fraction M_1 separated into 3 components, C_1, C_2, C_3, respectively.

(ii) Hydrocortisone reduces strongly tetrazolium, whereas fraction M_1 reduces tetrazolium weakly, and furthermore the reaction did not always occur.

(iii) The sulfuric acid spectrum of fraction M_1 lacked the characteristic peaks of hydrocortisone.

(iv) Fraction M_1 proved to be considerably more polar than hydrocortisone in the Bush B_5 system as well as in 95% ethyl acetate-5% benzene (TLC). The component with $R_{cortisol}$ 1.0 in that system could not have been hydrocortisone since it did not reduce tetrazolium on the plate.

3. Fraction M_2

a. Chromatographic Mobilities

Fraction M_2 moved slightly faster than cortisone in TPG, but slower than cortisone in the Bush B_5 system, and with the same speed in aldosterone (Fig. 9).

This fraction, after being separated in TPG from the other lipid fractions, was eluted and chromatographed in different thin-layer chromatography systems. Its mobility was compared to that of aldosterone, and in all cases proved to be identical with that of the standard (Table IX).

In the benzene-ethyl acetate-methanol system, fraction M_2 separated into three components of mobilities with respect to cortisone (R_E) of .50, .72, 1.0 respectively. The major component, which moved as

TABLE IX

MOBILITIES OF FRACTION M_2 AND OF SOME OF ITS DERIVATIVES

Compound	Paper Chromatography			Thin Layer Chromatography							Derivatives*	
	TPG	Bush B_5		20% IPA-Carb.Tet.[1]		BE[2]		BEM[3]			Acetylated	Acet.+Oxidiz.
	R_B	R_B	R_f	R_E	R_f	R_E	R_f	R_E			R_f	R_f
								C_1	C_2	C_3		
Fraction M_2	.40	.54	.45	.53	.16(+)	.40	.18(+)	.50	.72	1.0	.24	.40(+)
Aldosterone	-	.54	.45	.53	.16(+)	.40	.18(+)		.72		.25	.41(+)
Cortisone	.34	.64	.54		.29(+)		.44(+)					

Reaction with tetrazolium indicated in brackets.
TPG - Toluene-propylene glycol.
R_B - Mobility with respect to corticosterone.
R_E - " " " " cortisone.
R_f - " " " " the solvent front.
* - Derivatives chromatographed in benzene-propylene glycol.
1 - 20% isopropyl alcohol - 80% carbon tetrachloride.
2 - BE - 5% benzene - 95% ethyl acetate.
3 - BEM - benzene-ethyl acetate-methanol (74:15:11).

In BEM - fraction M_2 separated into 3 components, C_1, C_2, C_3, respectively.

aldosterone, (R_E .72), reduced tetrazolium and did not react with the Porter-Silber reagent, when eluted from the thin-layer plate. The eluate of the less polar component (R_E 1.0) gave a negative Porter-Silber reaction and reduced tetrazolium only very weakly, thus eliminating the possibility of its being cortisone. This was also the case with the most polar component (R_E .50).

b. Acetylation of Fraction M_2 and Oxidation of the Acetylated Product

The TPG fraction, M_2, after elution, was acetylated by Mattox's method for the selective acetylation of aldosterone at carbon-21. On chromatography it yielded a product with the mobility of standard aldosterone-21-monoacetate (Table IX).

The aldosterone acetate and the unknown acetate were oxidized with chromic acid and on chromatography, in the same system as above, yielded, in both cases, less polar reducing compounds with identical mobilities (Table IX).

c. Evidence that Fraction M_2 is Aldosterone

(i) Fraction M_2 had the same mobility as aldosterone in several different systems of chromatography.

(ii) Acetylation of fraction M_2 and the subsequent oxidation of the acetylated product yielded derivatives of fraction M_2 with mobilities identical to those of the corresponding aldosterone derivatives.

(iii) Fraction M_2 and aldosterone did not react with the

Porter-Silber reagent, both reduced tetrazolium. The sulfuric acid spectrum of both compounds exhibited a maximum around 290 mμ.

4. Fraction M_3

a. Chromatographic Mobilities

Fraction M_3 moved with the same mobility as corticosterone when chromatographed in toluene-propylene glycol, Bush B_5 (Fig. 9), and when subjected to thin-layer chromatography in benzene-ethyl acetate (Table X).

Fraction M_3, when acetylated with excess acetic anhydride and pyridine, yielded a product which moved with corticosterone acetate (Table X).

b. Sulfuric Acid Spectrum

The eluate of fraction M_3 gave a sulfuric acid spectrum identical to that of corticosterone, with maxima around 290, 330, 380 and 460 mμ, all characteristic of corticosterone (Fig. 11).

c. Infrared Spectrum

About 50 μg of crystalline fraction M_3 yielded an infrared spectrum that was the same as that of corticosterone in the analytical region (2.5-6.2 μ), and in the finger-print region, except between 6.2 and 7.2 μ. The discrepancy in this region might be due to the different modes of preparation of the samples for analysis. In the case of the reference steroid, the sample was evaporated from methanol and mixed

TABLE X

MOBILITIES OF FRACTION M_3 AND OF ITS ACETATE

Compound	Paper chromatography			Thin layer chromatography
	TPG	Bush B_5	CBPG	BE
	R_B	R_f	R_f	R_f
Fraction M_3	1.0(+)	.84		.25(+)
Corticosterone		.84		.25(+)
Fraction M_3 Acetate			.29(+)	
Corticosterone Acetate			.29(+)	

Reaction with tetrazolium indicated in brackets.

TPG - Toluene propylene-glycol.

CBPG - Cyclohexane-benzene (1:1) propylene-glycol.

BE - 5% Benzene - 95% ethyl acetate.

R_B - Mobility with respect to corticosterone.

R_f - Mobility with respect to the solvent front.

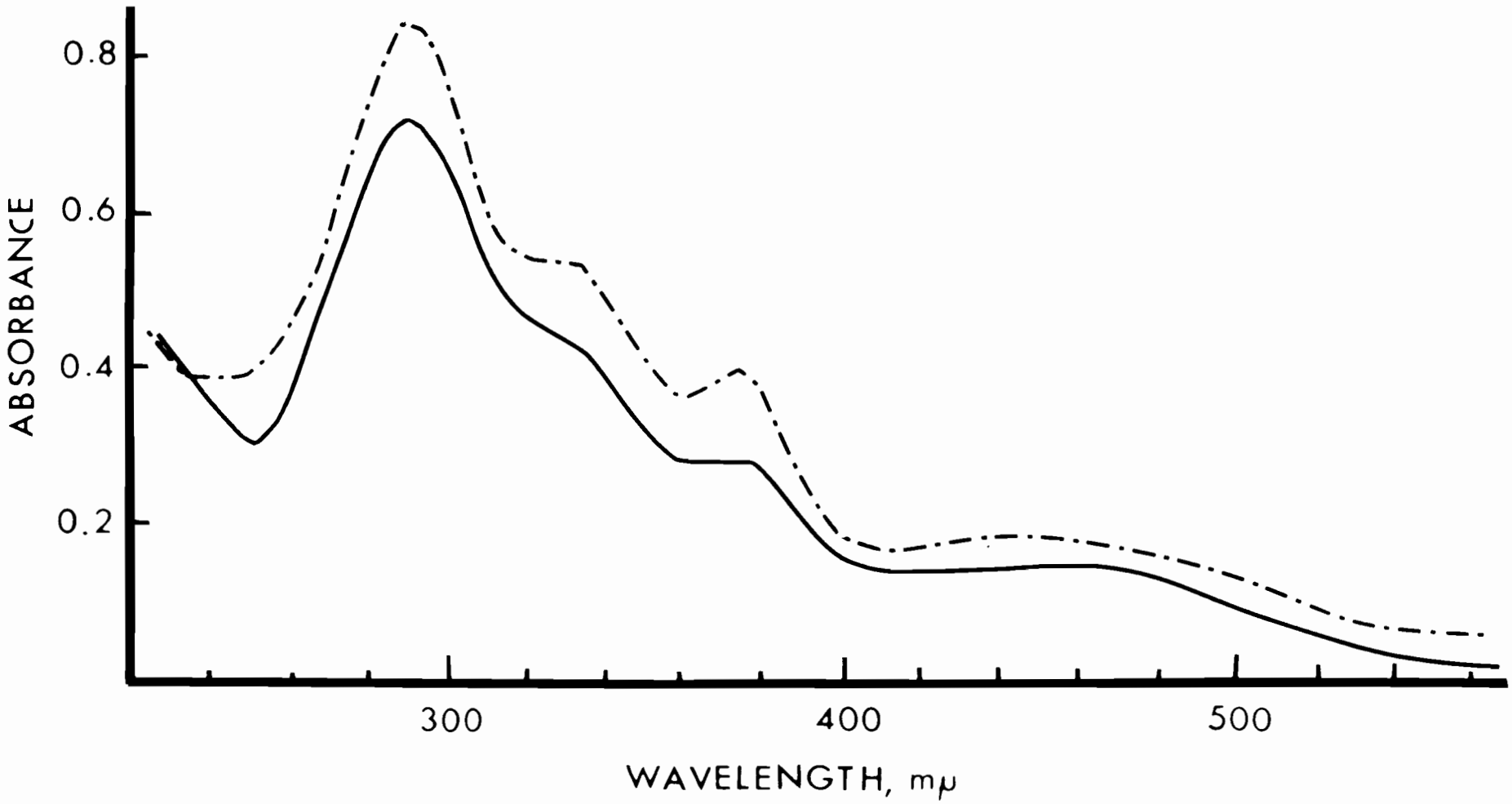


FIG. 11. Sulfuric acid spectrum of mouse adrenal fraction M_3. Broken line, standard corticosterone.

with potassium bromide, while in the case of the unknown, crystals were mixed with potassium bromide (Fig. 12).

d. Evidence that Fraction M_3 is Corticosterone

(i) This fraction had the mobility of corticosterone in three different systems of chromatography.

(ii) The mobility of its acetylated derivative was the same as that of acetylated corticosterone.

(iii) It reduced tetrazolium and did not react with the Porter-Silber reagent.

(iv) It gave a sulfuric acid spectrum characteristic of corticosterone.

(v) It gave an infrared spectrum corresponding to that of corticosterone, in the analytical region, and in the finger-print region above 7.2 μ.

5. Fractions Less Polar than Corticosterone

In order to achieve satisfactory separations of the two polar fractions, chromatograms were usually developed so that corticosterone was near the end of the paper. When all the material was allowed to remain on the strip, a reducing, ultraviolet-absorbing lipid with the mobility of 11-dehydrocorticosterone was obtained. As it was not eluted, no quantitative estimation of this fraction can be made. Visual examination of the area under the ultraviolet absorption scan of the

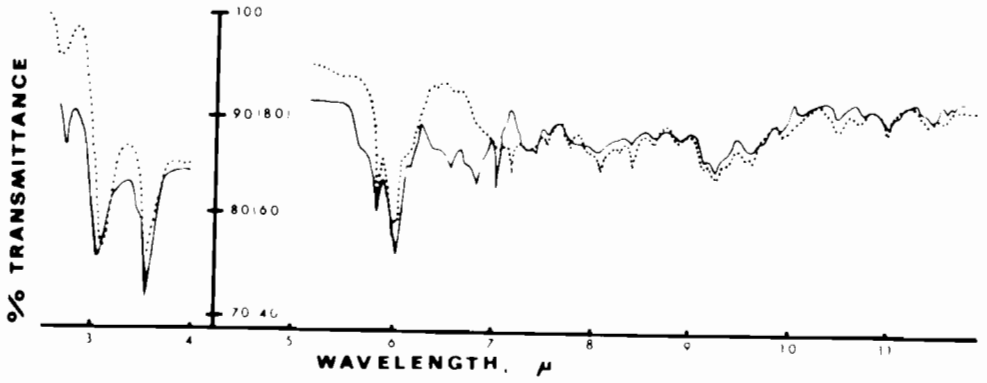


FIG. 12. Infrared spectrum of mouse adrenal fraction M_3. Broken line standard corticosterone.

paper strip, and of the intensity of the colour developed by tetrazolium, suggests that it contained the least amount of lipid among the four fractions.

No evidence of 11-deoxycorticosterone was obtained. The solvent front was ultraviolet-absorbing and could have included steroids less polar than 11-deoxycorticosterone. None of these fractions was eluted.

IV. DISCUSSION

1. Characterization of Steroid Fractions

The results of this study confirm the findings of other investigators in indicating that corticosterone is the predominant corticosteroid of the mouse adrenal. Corticosterone and 20α-dihydrocorticosterone were isolated from mouse plasma by Southcott et al. However, controversial literature exists concerning the production of 17-hydroxylated steroids by this species.

Several authors, among them Hofmann, Southcott et al. and Bloch and Cohen, agree that the mouse adrenal does not produce 17-hydroxycorticoids as major products. However, in a recent publication Varon and Touchstone report cortisol, and one other 17-hydroxylated compound as major components of the adrenal secretion of immature male mice. To explain the discrepancy of their results with those of other workers who used adult mice, they put forth the hypothesis that in mature male mice, androgens inhibit the formation of 17-hydroxylated compounds. However, even in their studies with mature mice, Varon and Touchstone report the presence of a fraction slightly more polar than cortisol, but also Porter-Silber positive, and hence assumed by these authors to be a 17-hydroxylated compound.

In the present study, fraction M_1 was not completely identified but it was shown to contain material with the mobility of 18-hydroxycorticosterone, as well as other components. In the Bush B_5 system, fraction M_1 moved with one-half the mobility of cortisol; it also failed

to react with the Porter-Silber reagent and its sulfuric acid spectrum lacked the characteristic peaks of cortisol and of 20α-dihydrocorticosterone. On the basis of these results, it was concluded that mouse adrenals do not produce cortisol or 20α-dihydrocorticosterone. The remaining components of fraction M_1 await further identification.

In the rat, the fraction which corresponds to mouse fraction M_2 has been identified as consisting mainly of aldosterone. This investigation supplied strong evidence that in the mouse too, the second most polar fraction in TPG is mainly aldosterone. In four out of five chromatographic systems this fraction moved as a single component, with aldosterone. It did not resolve upon acetylation, or oxidation of the acetylated product. The resolution of this fraction upon thin-layer chromatography in benzene-ethyl acetate-methanol was, therefore, unexpected. However, as the applied fraction represented the collection of many experiments, it is possible that it was converted to some tautomeric form of aldosterone. Cortisone could be excluded from fraction M_2, because of its failure to react with the Porter-Silber reagent, because of its different chromatographic mobilities and also because its sulfuric acid spectrum was different from that of cortisone.

The findings of this study indicate that the mouse does not elaborate 17-hydroxylated steroids. This is only the third mammalian species in which the absence of these compounds was demonstrated; the rabbit and the rat are the other two species evidently lacking an active adrenal 17-hydroxylase. Lack of 17-hydroxylated compounds in these

animals is unexpected and of unknown significance.

If androgens inhibit the formation of 17-hydroxylated compounds in the mouse, as postulated by Varon and Touchstone, it would be of interest to determine why androgens do not affect other species in a like manner. It has been recently shown that hyperplastic adrenals of young female mice are capable of producing active androgens. These abnormal adrenals also possess an active 17-hydroxylase. This evidence does not support the hypothesis of Varon and Touchstone that androgens inhibit the production of 17-hydroxylated compounds.

Quantitative changes in corticoid secretion have been shown to occur as the animal matures; the ratio of cortisol to corticosterone increases from 1.4 in ten-day old bull calves to 3.9 in mature bulls (144). This would suggest that androgens enhance the production of 17-hydroxycorticoids rather than inhibit it. The work of Varon and Touchstone awaits confirmation by other researchers.

The lack of 17-hydroxylated compounds does not always mean the absence of the 17-hydroxylase. Kass et al. (145) reported that administration of ACTH for one to four weeks to the rabbit, which normally does not produce 17-hydroxysteroids, resulted in large production of cortisol and a gradual decrease in the corticosterone output. These results would indicate that ACTH activated an already present, but inactive, 17-hydroxylating enzyme.

As indicated by the results of the present study, the mouse seems to produce the same corticoids as the rat, with the exception of

18-hydroxy-11-deoxycorticosterone. The absence of this compound was demonstrated by the lack of any Porter-Silber positive material among the adrenal lipids, as well as by the absence from chromatograms of any u.v.-absorbing area that might correspond to 18-hydroxy-11-deoxycorticosterone. Absence of this compound from the mouse is puzzling, if one considers that the mouse is capable of synthesizing 18-hydroxylated compounds. This deficiency may be due to an 11β-hydroxylase, more active in the mouse than in the rat.

In the mouse, as well as in the rat, corticosterone, aldosterone, and 18-hydroxycorticosterone were shown to be present. In addition to 18-hydroxycorticosterone, the most polar mouse fraction contained other material. This is in agreement with findings in the rat (Lucis et al. (146)); Birmingham et al. (unpublished data).

Although an oxygen function at C-18 is common among adrenal secretory products of many species, the rat is the only known animal to produce 18-hydroxy-11-deoxycorticosterone in major amounts. However, a compound that may represent 18-hydroxy-11-deoxycorticosterone has recently been detected in the camel adrenal and recently Carballeira and Venning obtained a compound with similar mobility as 18-hydroxy-11-deoxycorticosterone from human adrenals incubated with radioactive progesterone (147).

2. Control of Steroid Output

As in the rat, calcium was shown to be required for optimal response to ACTH. In the absence of both calcium and ACTH, very small

amounts of steroids were produced. Addition of ACTH, but not of calcium, increased the steroid output, suggesting that ACTH stimulates the steroid production, even in the absence of calcium although not maximally. During the second incubation period, with a fresh addition of ACTH, but without calcium, considerably more steroid was produced than in the first period. This might be due to a greater saturation of the gland with the hormone upon prolonged contact.

The role of calcium in steroid production in response to ACTH is not clear; however, it is known that calcium ions are not involved in the binding of ACTH by the adrenal cortex (87). Results obtained in the present study indicate that in the mouse the adrenal needs calcium in order to respond maximally to ACTH. Calcium is also needed for the basic steroid output, which occurs in the absence of ACTH.

The response of mouse adrenals to ACTH was proportional to the amount added, and not to the concentration of ACTH in the medium. The dose-response curve was the same whether ACTH was added in 10 ml or 2 ml of medium. From these results, it appears that the mouse adrenal cannot distinguish between different concentrations of ACTH, only between amounts. Somehow the gland is capable of collecting the hormone, with equal ease, from a large or small volume of medium. However, it is difficult to understand why a five-fold higher concentration of ACTH should not facilitate access of the hormone to its site of action, and hence be more effective than a dilute solution of the hormone. Birmingham and Kurlents demonstrated the same phenomenon with rat adrenals (62).

The mouse adrenal was shown to be considerably more responsive to ACTH than the rat adrenal. However, the endogenous production was the same in both glands. This is further proof of quantitative as well as qualitative differences between these two closely related species.

The dose-response curve for mouse adrenals did not level off, even at the highest concentration of ACTH that could be kept in solution. In the time studies, the rate of ACTH-stimulated steroid production dropped after four hours, while the endogenous production remained constant. An explanation for these observations might be that endogenous steroid substrate becomes limiting only after 200 µg of steroids per 100 mg adrenal have been produced. About this much was produced in four hours. However, even the highest concentration of ACTH did not produce this amount of steroids per 100 mg adrenal in two hours. Therefore, no fall-off in the dose-response curve might be expected, unless the incubation time was extended.

Stimulation by ACTH resulted in an increased steroid output. The greatest effect of ACTH was exerted on the production of corticosterone. Thus, in the first four hours of incubation, ACTH increased the production of corticosterone up to 12 times. During the same period there was a four-fold increase in fraction M_1 due to ACTH, while aldosterone production was increased only 2-3 times. Furthermore, fraction M_1 was consistently more affected by ACTH than was aldosterone, whereas inconsistent results were obtained in the relative amounts of the two polar fractions produced by the rat adrenals (69,121). ACTH had a more

marked effect on the production of corticosterone and of the two polar fractions, which include 18-hydroxycorticosterone and aldosterone, in the mouse adrenal than in the rat adrenal.

Corticosterone is known to be produced in all three zones of the adrenal cortex (148); ACTH has been shown to influence mainly the two inner zones (39,40,149). On the basis of these observations, stimulation by ACTH of corticosterone synthesis is to be expected.

Aldosterone has been shown to be produced in the zona glomerulosa. It has also been the accepted view that 18-oxygenation takes place only in this zone. Several investigators have reported that the zona glomerulosa is not under the influence of ACTH. However, the present investigation strongly suggests that ACTH, in vitro, stimulates the production of aldosterone as well as that of 18-hydroxycorticosterone in the mouse adrenal. This study is not the first evidence of ACTH stimulating the production of an 18-hydroxylated compound: ACTH increases the production of 18-hydroxy-11-deoxycorticosterone in the rat (126). However, the latter steroid is produced by the zona fasciculata-reticularis in this animal, providing evidence for an 18-hydroxylating system in the inner layers of the cortex, whereas aldosterone and 18-hydroxycorticosterone originate in the zona glomerulosa.

To explain the stimulatory effect of ACTH on fractions M_1 and M_2 one might conclude that either ACTH is capable of influencing the zona glomerulosa - assuming that this zone is indeed the site of elaboration of the two polar fractions in the mouse - or that the 18-

hydroxylating enzyme is present in the inner zones as well, and that the two polar fractions originate in the inner layers of the mouse adrenal. If it is accepted that aldosterone is produced only in the zona glomerulosa, then the present study offers evidence that ACTH is capable, in vitro, of stimulating the zona glomerulosa to an appreciable extent.

It is possible that the introduction of an oxygen function to form 18-hydroxycorticosterone is brought about by a different enzyme system from that responsible for the formation of aldosterone. This might account for the difference in the extent to which ACTH stimulated the production of the two polar fractions in the mouse.

V. SUMMARY

1. The mouse adrenal was shown to produce, *in vitro*, corticosterone, aldosterone, and polar fraction M_1, which contained 18-hydroxycorticosterone as well as other components.

2. No evidence for the production of 17-hydroxylated compounds was obtained, nor did the mouse adrenal produce 17-hydroxy-11-deoxycorticosterone.

3. Addition of ACTH caused an 8-fold increase in the production of corticosterone, a 2.7-fold increase in the production of fraction M_1 and a 1.9-fold increase in the production of aldosterone.

4. ACTH-stimulated steroid production decreased with incubation time, while the unstimulated steroid output remained constant throughout the incubation period.

5. The mouse adrenal required calcium for optimal response to ACTH.

6. Mouse adrenals are more responsive to ACTH than rat adrenals. Furthermore, the mouse adrenal, in its response to ACTH, does not distinguish between the concentration of ACTH in the medium, only between the total amount of ACTH present in the medium.

VI. BIBLIOGRAPHY

1. Chester Jones, I., The Adrenal Cortex, Cambridge University Press, 1957.

2. Chester Jones, I., Phillips, J.G., and Bellamy, D., Brit. Med. Bull., 18, 110, 1962.

3. Brown-Sequard, C., C.R. Acad. Sci., 43, 422, 1856.

4. Oliver, G., and Schafer, E.A., J. Physiol., 18, 230, 1895.

5. Biedl, A., The Internal Secretory Organs: Their Physiology and Pathology, Wm. Wood and Co., New York, 1913.

6. Baumann, E.J., and Kurland, S., J. Biol. Chem., 71, 287, 1927.

7. Marine, D., and Baumann, E.J., Am. J. Physiol., 81, 86, 1927.

8. Rubin, M.I., and Krick, E.T., Proc. Soc. Exper. Biol. Med., 31, 228, 1934.

9. Gaunt, J.H., Tobian, C.E., and Gaint, J.H., Am. J. Physiol., 111, 321, 1935.

10. Swingle, W.W., and Remington, J.W., Physiol. Rev., 24, 89, 1944.

11. Long, C.N.H., Katzin, B., and Fry, E.G., Endocrinology, 26, 309, 1940.

12. Lewis, R.A., Kuhlman, D., Delbue, C., Koepf, G.F., and Thorn, G.W., Endocrinology, 27, 971, 1940.

13. Thorn, G.W., Engel, L.L., and Eisenberg, H., J. Exptl. Med., 68, 1961, 1938.

14. Reichstein, T., and Shoppee, C.W., Vitamins and Hormones, Vol. I, Academic Press Inc., New York, 1943.

15. Simpson, S.A., Tait, J.F., Wettstein, A., Neher, R., v. Euw, J., Schindler, O., and Reichstein, T., Experientia, 10, 132, 1954.

16. Grattan, J.F., and Jensen, H., J. Biol. Chem., 135, 511, 1940.

17. Short, R.V., The Biosynthesis and Secretion of Adrenocortical Steroids, Cambridge University Press, 1960.

18. Beall, D., J. Endocrinol., 2, 81, 1940.

19. Beall, D., and Reichstein, T., Nature (London), 142, 479, 1938.

20. Ayres, P.J., Gould, R.P., Simpson, S.A., and Tait, J.F., Biochem. J., 63, 19P, 1956.

21. Ayres, P.J., Hechter, O., Saba, N., Simpson, S.A., and Tait, J.F., Biochem. J., 65, 22P, 1956.

22. Symington, T., The Biosynthesis and Secretion of Adrenocortical Steroids, Cambridge University Press, 1960.

23. Hechter, O., Jacobsen, R.P., Jeanloz, R.W., Levy, H., Marshall, C.W., Pincus, G., and Schenker, V., J. Am. Chem. Soc., 71, 3261, 1949.

24. Hechter, O., Zaffaroni, A., Jacobsen, R.P., Levy, H., Jeanloz, R.W., Schenker, V., and Pincus, G., Rec. Progr. Hormone Res., 6, 215, 1951.

25. Levy, H., Jeanloz, R.W., Marshall, C.W., Jacobsen, R.P., Hechter, O., Schenker, W., and Pincus, G., J. Biol. Chem., 203, 433, 1953.

26. Holtzbauer, M., J. Physiol., 139, 294, 1957.

27. Silber, R.H., Busch, R.D., and Oslapas, R., Clin. Chem., 4, 278, 1958.

28. Elliott, F.H., Birmingham, M.K., Schally, A.V., and Schonbaum, E., Endocrinology, 55, 721, 1954.

29. Smith, P.E., J. Am. Med. Assoc., 88, 158, 1927.

30. Smith, P.E., Am. J. Anat., 45, 205, 1930.

31. Yates, F.E., Urquhart, J., Physiol. Rev., 42, 359, 1962.

32. Wilson, H., Borris, J.J., and Bahn, R.C., Endocrinology, 62, 135, 1958.

33. Bergner, G.E., and Deane, H.W., Endocrinology, 43, 240, 1948.

34. Li, C.H., Evans, H.M., and Simpson, M.E., J. Biol. Chem., 149, 413, 1943.

35. Sayers, G., White, A., and Long, C.N.H., J. Biol. Chem., 149, 425, 1943.

36. Hofmann, K., Annual Rev. of Biochem., 31, 213, 1962.

37. Swann, H.G., Physiol. Rev., 20, 493, 1940.

38. Tepperman, J., Engel, F.L., and Long, C.N.H., Endocrinology, 32, 373, 1943.

39. Yoffey, J.M., and Baxter, J.S., J. Anat., 83, 89, 1949.

40. Stachenko, J., and Giroud, C.J.P., Endocrinology, 64, 743, 1959.

41. Deane, H.W., and Greep, R.O., Am. J. Anat., 79, 117, 1946.

42. Deane, H.W., Shaw, J.H., and Greep, R.O., Endocrinology, 43, 133, 1948.

43. Chester Jones, I., and Roby, C.C., J. Endocrinol. 10, 245, 1954.

44. Lostroh, A.J., and Woodward, P., Endocrinology, 62, 498, 1958.

45. Balfour, W.E., J. Physiol., 122, 59P, 1953.

46. Carpenter, R.K., McLeod, L.D., and Reiss, M., J. Physiol., 105, 231, 1946.

47. Saffran, M., Bayliss, M., and Webb, J.L., Federation Proc., 10, 116, 1951.

48. Haynes, R., Savard, K., and Dorfman, R.I., Science, 116, 690, 1953.

49. Gemzell, C., Acta Endocrinol., 1, Suppl. 1, 15, 1948.

50. Sayers, G., Sayers, M.A., White, A., and Long, C.N.H., Proc. Soc. Exptl. Biol. Med., 52, 200, 1943.

51. Sayers, G., Sayers, M.A., Liang, T., and Long, C.N.H., Endocrinology, 38, 1, 1946.

52. Selye, H., J. Clin. Endocrinol., 6, 117, 1946.

53. Sayers, G., Sayers, M.A., Lewis, H.L., and Long, C.N.H., Proc. Soc. Exptl. Biol. Med., 55, 238, 1944.

54. Hechter, O., and Pincus, G., Physiol. Rev., 34, 459, 1954.

55. Kersten, H., Leonhauser, S., and Staudinger, H., Biochim. et. Biophys. Acta, 29, 350, 1958.

56. Hedner, P., and Rerup, C., Acta Endocrinol., 41, 219, 1962.

57. Ingle, D.J., Li, C.H., and Evans, H.M., Endocrinology, 39, 32, 1946.

58. Saffran, M., Grad, B., and Bayliss, M., Endocrinology, 50, 639, 1952.

59. Haynes, R., Savard, K., Dorfman, R.I., J. Biol. Chem., 207, 925, 1954.

60. Macchi, I.A., and Hechter, O., Endocrinology, 55, 387, 1954.

61. Hayano, M., Saba, N., Dorfman, R.I., and Hechter, O., Rec. Progr. Hormone Res., 12, 79, 1956.

62. Birmingham, M.K., and Kurlents, E., Endocrinology, 62, 47, 1958.

63. Bush, I.E., Ciba Foundation Colloq. Endocrinol., 7, 210, 1953.

64. Holtzbauer, M., and Vogt, M., J. Physiol., 138, 449, 1957.

65. Schonbaum, E., and Saffran, M., Rev. Can. Biol., 13, 495, 1954.

66. Reich, E., and Lehninger, A.L., Biochim. et. Biophys. Acta, 17, 136, 1955.

67. Sayers, G., and Sayers, M.A., Rec. Progr. Hormone Res., 2, 81, 1948.

68. Stone, D., and Hechter, O., Arch. Biochem. Biophys., 51, 457, 1954.

69. Ward, P.G., Birmingham, M.K., Acta Endocrinol., 39, 110, 1962.

70. Hayano, M., Saba, N., Dorfman, R.I., and Hechter, O., Rec. Progr. Hormone Res., 12, 79, 1956.

71. Hechter, O., Vitamins and Hormones, 13, 293, 1955.

72. Eichhorn, J., Halkerston, I.D.K., Feinstein, M., and Hechter, O., Proc. Soc. Exptl. Biol. Med., 103, 515, 1960.

73. Hechter, O., and Lester, G., Rec. Progr. Hormone Res., 16, 139, 1960.

74. Kelly, T.L., Nielson, E.D., Johnson, R.B., and Vestling, C.S., J. Biol. Chem., 212, 545, 1955.

75. Glock, G.E., and McLean, P., Biochem. J., 56, 171, 1954.

76. Haynes, R.C., and Berthet, L., J. Biol. Chem., 225, 115, 1957.

77. Sutherland, E.W., and Rall, T.W., J. Am. Chem. Soc., 79, 3608, 1957.

78. Haynes, R.C., J. Biol. Chem., 233, 1220, 1958.

79. Haynes, R.C., Koritz, S.B., and Peron, F.G., J. Biol. Chem., 234, 1421, 1959.

80. Haynes, R.C., Sutherland, E.W., and Rall, T.W., Rec. Progr. Hormone Res., 16, 121, 1960.

81. Saffran, M., Brit. Med. Bull., 18, 122, 1962.

82. Koritz, S.B., Peron, F.G., J. Biol. Chem., 230, 343, 1958.

83. Kobayashi, S., Yago, N., Morisaki, M., Ichii, S., and Matsuba, M., Steroids, 2, 167, 1963.

84. Ferguson, J.J., J. Biol. Chem., 238, 2754, 1963.

85. Harding, B.W., Nelson, D.H., Endocrinology, 75, 501, 1964.

86. Harding, B.W., Nelson, D.H., Endocrinology, 75, 506, 1964.

87. Birmingham, M.K., Kurlents, E., Lane, R., Muhlstock, B., and Traikov, H., Can. J. Biochem. Physiol., 38, 1077, 1960.

88. Birmingham, M.K., Elliott, F.H., and Valere, P., Endocrinol., 53, 687, 1953.

89. Chavin, W., Kovacevic, A., Gen. and Comp. Endocrinol., 1, 264, 1961.

90. Idler, D.R., Ronald, A.J., and Schmidt, P.J., J. Am. Chem. Soc., 81, 1260, 1959.

91. Bern, H.A., de Roos, C.C., and Biglieri, E.G., Gen. and Comp. Endocrinol., 2, 490, 1962.

92. Carstensen, H., Burgers, A.C.J., and Li, C.H., Gen. and Comp. Endocrinol., 1, 37, 1961.

93. Kraulis, I., and Birmingham, M.K., Acta Endocrinol., 47, 76, 1964.

94. Ulick, S., Kusch, K., J. Am. Chem. Soc., 82, 6421, 1960.

95. Personen, S., and Rapola, J., Gen. and Comp. Endocrinol., 2, 425, 1962.

96. Macchi, I.A., Program of the 46th Meeting of the Endocrine Society, abstr. 187, 1964.

97. Sandor, T., Lamoureux, J., and Lanthier, A., Steroids, 4, 213, 1964.

98. Phillips, J.G., Chester Jones, I., and Bellamy, D., J. Endocrinol., 25, 233, 1962.

99. Sandor, T., Lamoureux, J., and Lanthier, A., Endocrinology, 73, 629, 1963.

100. de Roos, R., Gen. and Comp. Endocrinol., 1, 494, 1961.

101. Oliver, J.T., Peron, F.G., Steroids, 4, 351, 1964.

102. Chester Jones, I., Vinson, G.P., Jarrett, I.G., and Sharman, G.B., J. Endocrinol., 30, 149, 1964.

103. de Roos, C.C., and Bern, H.A., Gen. and Comp. Endocrinol., 1, 275, 1961.

104. Race, G.J., and Wu, H.M., Gen. and Comp. Endocrinol., 4, 199, 1964.

105. McDonald, I.R., and Reich, M., J. Physiol., 147, 33, 1959.

106. Bush, I.E., Ferguson, K.A., J. Endocrinol., 10, 1, 1953.

107. Chester Jones, I., Jarrett, I.G., Vinson, G.P., and Potter, K., J. Endocrinol., 29, 211, 1964.

108. Sandor, T., Lanthier, A., Acta Endocrinol., 42, 355, 1963.

109. Gassner, F.X., Nelson, D.H., Reich, H., Rapala, R.T., and Samuels, L.T., Proc. Soc. Exptl. Biol. Med., 77, 829, 1951.

110. Fajer, A., and Vogt, M., International Congress of Hormonal Steroids, abstr. 153, 1962.

111. Reich, H., Nelson, D.H., and Zaffaroni, A., J. Biol. Chem., 187, 411, 1950.

112. Farrell, G.L., Rauschkolb, E.W., and Royce, P.G., Am. J. Physiol., 182, 269, 1955.

113. Schindler, W.J., and Knigge, K.M., Endocrinology, 65, 739, 1959.

114. Romanoff, E.B., Hudson, P., and Pincus, G., J. Clin. Endocrinol. Metab., 13, 1546, 1953.

115. Sweat, M., J. Clin. Endocrinol. Metab., 15, 1043, 1955.

116. Hudson, P.B., and Lombardo, M.E., J. Clin. Endocrinol. Metab., 15, 324, 1955.

117. Ulick, S., and Kuschvetter, K., International Congress of Hormonal Steroids, abstr. 213, 1962.

118. Aires, M.M., Kadekaro, M., and Fajer, A., International Congress of Hormonal Steroids, abstr. 152, 1962.

119. Reif, A.E., and Longwell, B.B., Endocrinology, 62, 573, 1958.

120. Gassner, F.X., Nelson, D.H., Reich, H., Rapala, R.T., and Samuels, L.T., Proc. Soc. Exptl. Biol. Med., 77, 829, 1951.

121. Ward, P.G., and Birmingham, M.K., Biochem. J., 76, 269, 1960.

122. Bush, I.E., J. Physiol., 115, 12P, 1951.

123. Vogt, J., J. Physiol., 130, 601, 1955.

124. Giroud, C.J.P., Stachenko, J., and Venning, E.H., Proc. Soc. Exptl. Biol. Med., 92, 154, 1956.

125. Giroud, C.J.P., Saffran, M., Schally, A.V., Stachenko, J., and Venning, E.H., Proc. Soc. Exptl. Biol. Med., 92, 855, 1956.

126. Birmingham, M.K., and Ward, P.J., J. Biol. Chem., 236, 1661, 1961.

127. Peron, F.G., Endocrinology, 69, 39, 1961.

128. Peron, F.G., Program of the 43rd Meeting of the Endocrine Society, abstr. 82, 1961.

129. Hofmann, F.G., Endocrinology, 59, 712, 1956.

130. Southcott, C.M., Bandy, H.E., Newsom, S.E., and Darrach, M., Can. J. Biochem. Physiol., 34, 913, 1956.

131. Bloch, E., Cohen, A.I., J. Nat. Cancer Inst., 24, 97, 1960.

132. Varon, H.H., and Touchstone, J.C., Acta Endocrinol., 46, 65, 1964.

133. Huseby, R.A., and Domingues, O.V., Program of the 46th Meeting of the Endocrine Society, abstr. 75, 1964.

134. Vinson, G.P., and Chester Jones, I., J. Endocrinol., 29, 185, 1964.

135. Raman, P.B., Ertel, R.J., and Ungar, F., Endocrinology, 74, 865, 1964.

136. Saffran, M., and Schally, A.V., Endocrinology, 56, 523, 1955.

137. Bush, I.E., and Willoughby, M., Biochem. J., 67, 689, 1957.

138. Burton, R.B., Zaffaroni, A., and Keutmann, E.H., J. Biol. Chem., 188, 763, 1951.

139. Bush, I.E., Biochem. J., 50, 370, 1952.

140. Silber, R.H., and Porter, C.C., J. Biol. Chem., 210, 923, 1954.

141. Bernstein, S., and Lenhard, R., J. Org. Chem., 18, 1146, 1953.

142. Zaffaroni, A., and Burton, R.B., J. Biol. Chem., 193, 749, 1951.

143. Mattox, V.R., Mason, H.L., and Albert, A., J. Biol. Chem., 218, 359, 1956.

144. Balfour, W.E., Brit. Med. Bull., 18, 114, 1962.

145. Kass, E.H., Hechter, O., Macchi, I.A., and Mou, T.W., Proc. Soc. Exptl. Biol. Med., 85, 583, 1954.

146. Lucis, O.J., Dyrenfurth, I., and Venning, E.H., Can. J. Biochem. Physiol., 39, 901, 1961.

147. Carballeira, A., and Venning, E.A., Steroids, 4, 329, 1964.

148. Stachenko, J., and Giroud, C.J.P., Endocrinology, 64, 730, 1959.

149. Sonenberg, M., Keston, A.S., and Money, W.L., Endocrinology, 48, 148, 1951.