BIOSYNTHESIS OF STEROIDS BY THE RAT ADRENAL USING RADIOACTIVE PRECURSORS

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NOMENCLATURE OF C_{21} STEROIDS

Trivial or Abbreviated Name	Systematic Name
19-OH-S	17α,19,21-trihydroxy-pregn-4-ene-3,20-dione
6β-OH-F	6β,11β,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione
6β-OH-E	6β,17α,21-trihydroxy-pregn-4-ene-3,11,20-trione
16α-OH-B	11β,16α,21-trihydroxy-pregn-4-ene-3,20-dione
18-OH-B	11β,18,21-trihydroxy-pregn-4-ene-3,20-dione
6β-OH-S	6β,17α,21-trihydroxy-pregn-4-ene-3,20-dione
19-OH-B	11β,19,21-trihydroxy-pregn-4-ene-3,20-dione
Cortisol (F)	11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione
Aldosterone	11β,21-dihydroxy-pregn-4-ene-3,20-dione-18-al
Cortisone (E)	17α,21-dihydroxy-pregn-4-ene-3,11,20-trione
19-OH-DOC	19,21-dihydroxy-pregn-4-ene-3,20-dione
18-OH-DOC	18,21-dihydroxy-pregn-4-ene-3,20-dione
6β-OH-DOC	6β,21-dihydroxy-pregn-4-ene-3,20-dione
11-deoxy-cortisol (S)	17α,21-dihydroxy-pregn-4-ene-3,20-dione

NOMENCLATURE OF C_{21} STEROIDS (continued)

Corticosterone (B)	11β,21-dihydroxy-pregn-4-ene-3,20-dione
16α-OH-prog.	16α-hydroxy-pregn-4-ene-3,20-dione
11-dehydrocorticosterone (A)	21-hydroxy-pregn-4-ene-3,11,20-trione
Dihydro-A	21-hydroxy-5α-pregnane-3,11,20-trione
THA	3α,21-dihydroxy-5β-pregnane-11,20-dione
6β-OH-prog.	6β-hydroxy-pregn-4-ene-3,20-dione
11β-OH-prog.	11β-hydroxy-pregn-4-ene-3,20-dione
17α-OH-prog.	17α-hydroxy-pregn-4-ene-3,20-dione
18-OH-prog.	18-hydroxy-pregn-4-ene-3,20-dione
11-deoxy-corticosterone (DOC)	21-hydroxy-pregn-4-ene-3,20-dione
	20β-hydroxy-pregn-4-ene-3-one
11-keto-Prog.	Pregn-4-ene-3,11,20-trione
17α-OH-pregnenolone	3β,17α-dihydroxy-pregn-5-ene-20-one
Pregnenolone	3β-hydroxy-pregn-5-ene-20-one
Progesterone	Pregn-4-ene-3,20-dione
16-dehydro-prog.	Pregn-4,16-diene-3,20-dione

NOMENCLATURE OF C_{19} STEROIDS

Trivial or Abbreviated Name	Systematic Name
19-OH-testosterone	17β,19-dihydroxy-androst-4-ene-3-one
19-OH-androstenedione	19-hydroxy-androst-4-ene-3,17-dione
	3β,11β-dihydroxy-5α-androstane-17-one
	3β,17β-dihydroxy-androst-5-ene
DHEA	3β-hydroxy-androst-5-ene-17-one
11β-OH-androstenedione	11β-hydroxy-androst-4-ene-3,17-dione
Testosterone	17β-hydroxy-androst-4-ene-3-one
Adrenosterone	androst-4-ene-3,11,17-trione
Epitestosterone	17α-hydroxy-androst-4-ene-3-one
19-aldo-androstenedione	androst-4-ene-3,17-dione-19-al
Etiocholanolone	3α-hydroxy-5β-androstane-17-one
Androsterone	3α-hydroxy-5α-androstane-17-one
Androstenedione-	androst-4-ene-3,17-dione

NOMENCLATURE OF C_{18} STEROIDS

Trivial or Abbreviated Name	Systematic Name
Estriol	3,16α-17β-trihydroxy-estra-1,3,5(10)-triene
2-OH-estradiol-17β	2,3,17β-trihydroxy-estra-1,3,5(10)-triene
16-epiestriol	3,16β,17β-trihydroxy-estra-1,3,5(10)-triene
16-keto-estrone	3 -hydroxy-estra-1,3,5(10)-triene-16,17-dione
Paraquinol	10β,17β-dihydroxy-estra-1,4-diene-3-one
16-keto-estradiol-17β	3,17β-dihydroxy-estra-1,3,5(10)-triene-16-one
16α-OH-estrone	3,16α-dihydroxy-estra-1,3,5(10)-triene-17-one
Estradiol-16α	3,16α-dihydroxy-estra-1,3,5(10)-triene
Estradiol-16β	3,16β-dihydroxy-estra-1,3,5(10)-triene
Estradiol-17β	3, 17β-dihydroxy-estra-1,3,5(10)-triene
Estradiol-17α	3, 17α-dihydroxy-estra-1,3,5(10)-triene
Estrone-16-keto	3-hydroxy-estra-1,3,5(10)-triene-16-one
Estrone	3-hydroxy-estra-1,3,5(10)-triene-17-one.

NOMENCLATURE OF OTHER COMPOUNDS

Trivial or Abbreviated Name	Systematic Name
19-nortestosterone	17β-hydroxy-19-nor-androst-4-ene-3-one
19-norandrostenedione	19-nor-androst-4-ene-3,17-dione
Bisdehydrodoisynolic acid	
Aromatized progesterone	3β-hydroxy-19-nor-pregn-1,3,5(10)-triene-20-one

I. REVIEW OF LITERATURE

1. Introduction:

Mammalian adrenal cortex morphologically is arranged into three zones: the outer layer, called zona glomerulosa, the middle layer where cells are arranged in more radial fashion called zona fasciculata and the innermost layer bordering the medulla is called zona reticularis. This classification originates with the studies of Arnold (1) in 1866.

Embryologically the adrenal cortex develops from coeloma epithelial tissue and the adrenal medulla from differenciated ectoderm - sympathetic nervous tissue.

The vital importance of adrenal glands in the physiology of the organism was first demonstrated by Brown-Sequard in 1856 (2), who showed that bilateral adrenalectomy in animals was fatal.

Further experimental studies on cortical function were resumed only in 1929 when Swingle and Pfiffner (3) demonstrated that adrenalectomized animals can be maintained on injections of adrenocortical extracts. A year later, Smith (4) showed that the adrenal cortex is under the influence of the pituitary gland.

The research on the hormones of the adrenal cortex is based on the following facts as outlined by Reichstein and Schoppee (5):

(a) The adrenals are vital organs in nearly all animals. Complete bilateral adrenalectomy leads to death.

(b) The vital function is connected with the adrenal cortex, and appears to operate by delivery into the blood of a mixture of substances, since by injection of suitable cortical extracts, adrenalectomized animals can be kept alive and the numerous insufficiency symptoms prevented or cured.

(c) Investigations of active cortical extracts show that the activity can be concentrated in those fractions which contain principally a mixture of relatively heavily oxygen substituted steroids.

The most important deficiency symptoms which follow adrenalectomy and which are susceptible of quantitative estimation are:

(a) Disturbances of the Na^+, K^+, Cl^- and water balance with increased excretion of sodium and chlorine ions, and water, and a retention of K^+.

(b) Increase of the urea content of the blood.

(c) Asthenia.

(d) Disturbance of carbohydrate metabolism with decrease in liver glycogen and diminished resistance to insulin.

(e) Reduction of resistance to various traumata like cold, mechanical or chemical shock.

Between 1930 and 1940, a number of steroids were isolated from adrenal extracts by Reichstein, Kendall and Wintersteiner (5).

Most of the steroids isolated were derivatives of the allopregnane and the Δ^4-pregnene series. A few belonged to androstane series. At that time it was noted that the biological activity of the steroids is associated with a Δ^4 double bond. It was recognized that substitutions at carbon 11, 17, and 21 altered the biological properties and the reduction of 20-keto group resulted in loss of activity. During the early work of Reichstein, of all the pure steroids examined the nearest qualitative approach to the full activity of adrenal cortical extract in rats was shown by corticosterone. The high potency of corticosterone in rat assays may be attributed to the fact that corticosterone is the principal steroid secreted by the rat adrenal cortex.

After crystallization of all the known steroid hormones from adrenocortical extracts, there remained an amorphous fraction, which had a marked sodium retaining effect. It was predicted by Reichstein that this fraction should contain compounds of $C_{21}O_5$ group (5). A further purification and separation of the amorphous fraction was not possible until after 1950, when paper chromatographic methods were developed for the steroids. In 1952, Simpson and Tait (6, 7) isolated from the amorphous fraction the active principle-aldosterone.

2. Steroid hormone biosynthesis by rat adrenal glands:

The rat has been used as a principal animal for hormone assays since early 1930. Studies of the biosynthesis of

steroid hormones in this animal originate with Bush (8), who showed that rat adrenal glands secrete corticosterone. This was confirmed by Singer and Stack-Dunne (9).

Saffran and Schally (10) in 1955 demonstrated that rat adrenal quarters incubated in vitro synthesize ultraviolet light absorbing steroids, which increase in the presence of corticotropin. The increase of the steroid hormone synthesis is proportional to the logarithm of the ACTH dose. This method has been used for the assay of corticotropin.

Studies of Giroud et al (11,12) with rat adrenal tissue in vitro reveal that some 7 ultraviolet light absorbing compounds separable by Bush B_5 paper chromatographic system are produced. Out of these, aldosterone and corticosterone were identified. Numerous workers have confirmed that corticosterone is the main steroid secreted by the rat adrenal gland in vivo and in vitro (13, 14, 15, 16, 17, 18).

So far, none of the investigators working with rat adrenal glands could demonstrate the presence of 17α-hydroxylated steroids. Several authors (19, 13) have shown, however, that rat adrenal glands secrete in vitro appreciable amounts of material which reacts with phenylhydrazine sulfuric acid reagent of Silber and Porter. This reaction has been regarded as an indicative for 17α-hydroxy steroids. Recent work of Birmingham and Ward (20) has shown that the phenylhydrazine reactive compound is 18-OH-DOC. Peron (15) has isolated

18-OH-B. Apart from these steroids, the secretion of DOC and compound A by rat adrenal cortex have been observed (21, 22).

3. Functional zonation:

Investigations of Deane and Greep (23, 24) have shown that the adrenal glomerulosa participates in metabolism of electrolytes. In vitro incubation of beef adrenal zona glomerulosa by Ayres et al (25) and incubation of rat adrenal capsules by Giroud et al (11) showed that the surviving zona glomerulosa cells secreted aldosterone, whereas the inner zones did not form aldosterone. Besides aldosterone, rat adrenal capsules also secrete in vitro 18-OH-B and corticosterone (11, 14, 21). The secretion of aldosterone and corticosterone by rat adrenal capsules in vitro is increased by corticotropin and posterior pituitary extract (14, 26). The increase in corticosterone secretion in these preparations parallels that of aldosterone, whereas in whole adrenal preparations the response in corticosterone is far greater.

Further studies on functional zonation of the rat adrenal gland have been done by Sheppard et al (21). These authors, using progesterone-^{14}C confirmed that rat adrenal capsules and decapsulated glands contain enzymes capable of hydroxylation of the steroid molecule at the 21, 18 and 11 positions. Aldosterone and DOC originate almost entirely from the capsular portion of the gland containing cells of the

zona glomerulosa.

It was estimated that ACTH increases 3.5 times the production of corticosterone and 1.5 times that of aldosterone by the capsular portion of the adrenal. The incorporation of labelled progesterone precursor in these two compounds in presence of ACTH decreases. This is explained by the authors (21), that ACTH in the adrenal tissue increases the available progesterone pool by increased breakdown of endogenous cholesterol, thus lowering the specific activity of progesterone available for further hydroxylation.

Progesterone-^{14}C by rat adrenal capsules containing zona glomerulosa cells is converted to 9 compounds, whereas decapsulated adrenal gland produced 10 compounds. The authors claim that the whole adrenal cortex forms from progesterone at least 11 conversion products (21).

4. In vivo secretion and distribution of steroids:

Levels of free corticosteroids in rat plasma have been measured by Guillemin et al (16) using the method based on fluorescence of compound B in sulphuric acid. These authors found that the resting levels in peripheral plasma for adult male rats range from 9 to 15 µg/100 ml. After stress the circulating free plasma corticosteroids increase up to five fold. The plasma corticosteroid levels in hypophysectomized animals range from 3.5 to 6.5 µg/100 ml. The U.S.P. corticotropin standard on intravenous administration

to rats, hypophysectomized 24 hours prior to assay, increases the plasma corticosteroids. In a dose range from 0.1 to 1.0 milliunits of ACTH the corticosteroid levels rise following a linear log-dose response. Hypophysectomized rats do not respond to purified FSH, LH, TSH, STH, MSH and arginine vasopressin with increases in plasma corticosteroids.

Cortes et al (27) have compared the plasma levels of free 18-OH-DOC and corticosterone in normal and hypophysectomized rats utilizing the Porter-Silber colorimetric reaction for 18-OH-DOC and fluorimetry for corticosterone. In plasma of arterial blood and also adrenal venous blood the concentration of 18-OH-DOC is about one half that of the corticosterone. After hypophysectomy these two hormones are found to be in equal amounts. On treatment with ACTH, the concentration of 18-OH-DOC and corticosterone increases 5 to 12 fold in the adrenal venous plasma of hypophysectomized animals.

In normal female rats, the plasma free 18-OH-DOC and corticosterone are found to be greater than in males. The sex differences in corticosterone secretion have been observed by Glenister and Yates (28), who suggested that the female rat adrenal cortex secretes corticosterone at a higher rate than that of the male. Also Kitay (29) observed that the plasma corticosterone level in female adrenal vein blood is some 2.5 times higher than that found in male rats.

Glenister and Yates (28) explain the increased corticosterone secretion in female rats on the grounds of increased Δ^4 steroid dehydrogenase activity in the liver, which leads to a greater rate of inactivation of adrenal cortical hormones in females than in males. The estimated half life of exogenous labelled corticosterone in female rats is found to be 11.7 and 14.3 minutes which is shorter than that observed in males (18.2 and 21.6 minutes). The female adrenal glands are larger in size than those in males. The greater secretion rate of corticosteroids by female adrenal glands presumably requires an increased output of ACTH or a higher sensitivity of the female adrenal to ACTH.

There are experimental observations reported concerning the effects of estrogens on the adrenal cortex. Estradiol in male rats increases the adrenal weight but does not change the weight in female rats (30). In vitro adrenal glands from male rats and castrated female rats increase the biosynthesis of steroid hormones in the presence of estradiol (30). These observations indicate that estrogens play a part in adrenocortical physiology.

Aldosterone has been isolated from the rat adrenal venous plasma by Singer and Stack-Dunne (9).

The secretion rate of aldosterone in rats by the double isotope derivative method has been recently reported by Eilers and Peterson (31). Normal rats secrete 2.7 mμg/

min. whereas corticosterone is secreted at a rate of 1.0 µg/min. Hypophysectomy reduces the secretion of aldosterone to 1.9 mµg/min and nephrectomy to 1.6 mµg/min. After both hypophysectomy and nephrectomy, the secretion of aldosterone declines to 0.9 mµg/min. In sodium deficient rats, aldosterone secretion was 5.5 mµg/min: after hypophysectomy the secretion rate remained elevated after hypophysectomy and nephrectomy. Also, induction of hyperkalemia increases aldosterone secretion in hypophysectomized rats. In normal rats, the constriction of the superior vena cava elevates aldosterone secretion; this response could not be provoked after nephrectomy. Infusion of crude kidney extract and synthetic angiotensin II in normal rats failed to show an increased output of aldosterone. The authors conclude that regulation of aldosterone in rat is similar to that of other mammals with exception in the response to crude kidney extract and angiotensin II.

Infusion of angiotensin II into the jugular vein of hypophysectomized rats with ligated renal blood vessels as shown by Singer (32) generally results in an increase in aldosterone secretion. The effect on corticosterone secretion was small and physiologically insignificant. Chronic injections of angiotensin II to normal male rats as shown by Glaz and Sugar (33), contrary to the observations in vivo by Eilers and Peterson (31), increases the capacity

of the adrenal glands to synthesize aldosterone in vitro with only minor increases in corticosterone production.

Injections of growth hormone, derived from various species, to hypophysectomized rats increase the biosynthesis of aldosterone with a minor effect on corticosterone demonstrable by in vitro incubations of the adrenal glands (34, 35).

The release of corticosterone into adrenal venous blood in response to stress and ACTH administration as shown by Slusher and Roberts (36), is not an immediate one. Corticosterone response reaches its maximum between 15 and 30 minutes after stimulus. The adrenal ascorbic acid depletion preceeds the corticosterone response and is completed within the first 15 minutes. Ascorbic acid depletion and steroid hormone biosynthesis, apparently, are two different events caused by ACTH. Recently Paquet et al (37), using a mathematical approach for the study of hypophysectomized rat adrenal response to corticotropin, showed that the adrenal corticosterone concentration is the result of two factors with different time characteristics: production of the hormone by the gland and discharge into the blood stream. The half life of exogenous ACTH in these animals is calculated to be 50 seconds.

Corticosterone in rat plasma is bound to transcortin and albumin (38). The binding affinity of transcortin for corticosterone is markedly diminished by thyroidectomy and

enhanced by thyroxin administration (39). The basal secretion rate of corticosterone and the corticosteroidogenic response to stress is diminished in thyroidectomized rats (39). Administration of thyroxin increases both these parameters of adrenal activity (39). Steinetz and Beach (40) have shown that thyroid hormone administration increases total plasma concentration with a smaller volume of distribution for corticosterone. In light of the findings of Lambrie et al (39) this phenomenon is due to an enhanced binding of corticosterone by plasma proteins.

Information concerning the plasma binding of the second major steroid hormone, 18-OH-DOC, in the rat is not available.

The distribution of d-aldosterone-1,2-^{3}H after subcutaneous administration to female rats was studied by Grange and Gornall (41). A fairly constant level of radioactivity in plasma was found during the first hour after injection declining to low levels by 4 hours. The whole blood contained slightly more radioactivity than the plasma at all times.

Excretion of radioactivity in the bile and an apparent reabsorption by the small intestine was observed.

5. Rat adrenal regeneration:

Following enucleation of rat adrenal gland, the

adrenal parenchymal cells regenerate from the surviving zona glomerulosa cells and within a few weeks differentiate into nearly normal pattern of the adrenal zones. These regenerated adrenal glands have an impaired capacity of production of aldosterone and corticosterone both in vivo and in vitro. The impaired steroid hormone production is attributed to some enzymatic defects and decreased availability of steroid hormone precursors (42, 43).

Studies with labelled thymidine in normal rats show that the adrenal gland belongs to expanding tissues where new cells are permanently added to the cell population (44). Rat adrenal cortex posesses however a potential of regeneration.

An important observation noted after enucleation is that the regenerated adrenal cortex not only histologically differentiates but also leads to a reestablishment of functional zonation similar to that of a normal adrenal cortex. This would imply that the regenerated cells in the course of differentiation alter their enzymatic activities. Similar to the normal gland, aldosterone is formed only in the zona glomerulosa of the regenerated adrenal and the innermost zones do not contain demonstrable 18-oxydase activity, even though these zones apparently originated from the cells of the zona glomerulosa (42, 43).

6. Species differences in corticosteroid secretion:

Variations between cortisol and corticosterone secretion have been observed in different animal species and these can be separated into mainly cortisol producers and predominantly corticosterone producers (45). The physiological significance of this variation may be due to environmental factors. Bush (46) suggests that the enzymatic systems in the adrenal cortex is governed by genetic factors. However, the recent work of Varon and Touchstone (47) demonstrate in mice, which belong to the corticosterone producers, the biosynthesis of cortisol in adrenal glands of immature animals. The 17α hydroxylase activity diminishes with increasing age. Also testosterone administration to young mice changes the steroid hormone pattern toward corticosterone secretion, suggesting suppression of 17α hydroxylase.

Recent work of Huseby and Dominguez (48) with castrated BALB/C female mice shows that in these animals castration produces adrenal hyperplasia with evidence of production of androgenic steroids. In vitro, these adrenal glands convert pregnenolone to Δ^4-androstendione and testosterone. Progesterone precursor yields 17α-hydroxy-progesterone confirming the presence of 17α hydroxylase.

In rabbits, which belong to the predominant cortico-

sterone producers, a prolonged ACTH administration induces the secretion of cortisol (49). The secretion of cortisol by the normal rat adrenal gland has not yet been demonstrated.

In 1955, several groups independently described the in vitro 19 hydroxylation of DOC by beef adrenal preparations (50, 51). Mattox (52) isolated 19-OH-DOC directly after extraction of beef adrenal glands. Studies of Griffith (53) with adrenal glands from various species show that adrenals from golden hamster convert exogenous DOC to 19-OH-DOC to an extent of 30%. Incubation of adrenal tissue preparations from ox, sheep and goat yield also 19-OH-DOC, the conversion however is considerably less in these species.

The physiological action of 19-OH-DOC is not clear. This steroid has a weak sodium retaining activity in adrenalectomized rats - about 4% of that of DOC acetate; and in the Ingle work test its activity is less than 2% of cortisol (54).

Recent studies by Oliver and Peron (55) on mongolian gerbil, which is a rodent of the family of muridae (mice), show that the adrenal glands of this species contain also 19 hydroxylase. From the adrenal venous blood of these animals, two major steroids, cortisol and 19-OH-S, were isolated in equal amounts. Aldosterone and an unidentified compound (X_2), less polar than cortisone, were also isolated. Incubation of adrenal quarters with labelled progesterone results in the formation of 19-OH-S and

compound X_2 in addition to the expected corticosteroids. The biological function of 19-OH-S in these animals is not clear. This compound may be involved in water and electrolyte regulation in these desert animals. Burns (56) has shown that these animals can survive for 37 days on a diet of dry barley. On this dry diet the gerbils excrete highly concentrated urine containing up to 21% of urea and 9.4% of total electrolytes.

Human fetal adrenal glands also contain steroid 19 hydroxylase, the activity of this enzyme in adult human adrenal tissue could not be detected (53). The presence of 19 hydroxylase in the adrenal cortex is of a considerable interest.

Several authors (57, 58) have demonstrated that the aromatization of ring A of testosterone and Δ^4-androstenedione may proceed via 19 hydroxylation and a subsequent removal of the C-19 fragment as formaldehyde. It has been suggested that adrenal gland is capable of producing estrogens. Beall (59) isolated estrone from beef adrenal glands by extraction. Also pathological human adrenal tissues form in vitro various estrogens (60, 61). The presence of 19 hydroxylase in rat adrenal cortex has been recently reported (62) and is described in this thesis. Formation of estrogens by rat adrenal cortex has not been demonstrated.

Recent findings of Ryan et al (63) show that the urine of castrated rats contains 17 ketosteroids, which is of considerable importance. Adult castrated Sprague-Dawley rats excrete 12 ± 1 µg/24 hrs. 17 ketosteroids in terms of dehydroepiandrosterone standard. The 17 ketosteroid level increases on administration of ACTH. It is difficult to visualize the endogenous formation of 17 ketosteroids in the absence of 17α hydroxylase in the adrenal cortex. Dorfman (64) has suggested the existance of an alternate pathway from progesterone to testosterone via testosterone acetate. This pathway, apparently, operates in human polycystic ovarian tissue. The formation of testosterone acetate directly from progesterone is based mainly on theoretical interpretations. Testosterone acetate as such has not been isolated. This theory, however, may explain the origin of 17 ketosteroids in urine of castrated rats.

Studies on the ultra structure of the rat adrenal by Nishikawa et al (65) reveal some findings which may be related to the sex hormone production. Chorionic gonadotropin administration to normal and castrated rats exerts a stimulating effect on cells of the inner layer of the zona fasciculata and outer part of the zona reticularis, particularly in alterations of mitochondria. The zona

reticularis of the adrenal cortex is often referred to as the sexual zone (65). In the mouse adrenal the x-zone which belongs to the zona reticularis is influenced by castration and androgenic hormone administration (66,67). In rats the x-zone is not apparent under light microscopy (67).

7. Regulation of biosynthesis of steroid hormones:

The complete sequences of reactions involved in the biosynthesis of adrenal steroids are not fully known. In 1951, Haines and co-workers (68) demonstrated incorporation of ^{14}C-acetate into steroid molecules by pig adrenal glands in vitro. Haynes, Savard and Dorfman (69) confirmed this finding with cow adrenal slices. In perfusion experiments with bovine adrenal glands it was shown by Stone and Hechter (70) that ^{14}C-labelled acetate, cholesterol and progesterone give rise to labelled corticosteroids. In vivo administration of sodium acetate-1-^{14}C to a pregnant mare as shown by Heard et al (71) leads to the excretion of ^{14}C-labelled estrogens in urine, indicating that the acetate molecule is also incorporated into phenolic steroids. Studies of labelled acetate metabolism in cell free hog adrenal preparations reveal that this system incorporates acetate into progesterone and corticosteroids but not into cholesterol (72). Whether cholesterol is an obligatory intermediate in the adrenal cortical biosynthesis is still a controversial question. On feeding cholesterol-^{14}C

to guinea pigs Werbin and Chaikoff (73) showed that about 60% of the adrenal cholesterol is derived from plasma. Cortisol excreted in the urine had the same specific activity as the adrenal cholesterol, thus indicating that the total cholesterol pool can be drawn for cortisol biosynthesis in this species.

The mechanism of ACTH action on the adrenal cortex has been studied by a number of investigators. In perfused bovine adrenal glands as shown by Stone and Hechter (70) ACTH does not significantly increase the incorporation of acetate into corticosteroids. Also the conversion of labelled progesterone by ACTH is influenced only to a minor degree. The conversion of cholesterol to corticosteroids is markedly enhanced by ACTH (70, 74). This led to the conclusion that ACTH acts in the perfused adrenal gland at the step between the conversion of cholesterol to Δ^5-pregnenolone which is rate limiting for steroid hormone formation.

The enzyme systems involved in these reactions (75) are associated with adrenal mitochondria. ACTH, apparently, accelerates 20β,22-dihydroxycholesterol transformation to Δ^5-pregnenolone by cleavage of the side chain between C-21 and C-22 of cholesterol, liberating isocaproic aldehyde (76).

Haynes and co-workers (77, 78) proposed another

mechanism of ACTH action which involves accumulation of 3',5'-adenosine monophosphate and activation of phosphorylase. The net result of these events is an increased level of intracellular glucose-6-phosphate from the adrenal glycogen which by further metabolism through hexose monophosphate shunt generates increased amounts of $NADPH_2$ necessary for steroid hormone hydroxylation. This theory is supported by experiments of Peron (79), who showed that rat adrenal glands in vitro maximally stimulated with ACTH, $NADPH_2$ and 3',5'-AMP bring about a synthesis of the same amounts of corticosteroids.

Studies of Koritz and Peron (79, 80) have further suggested that ACTH in vitro activate other enzyme systems in the adrenal gland and also can make enzymatically available a steroid precursor which is in adrenal tissue in a bound form. In beef adrenal cortex the basal levels of phosphorylase in the zona glomerulosa and in the zona fasciculata are similar (81). In vitro stimulation of phosphorylase by ACTH is demonstrable only in the zona fasciculata preparations (81). These observations are consistent with studies of Cohen (82), who showed that glycogen is diminished to a much greater extent in the zona fasciculata than in the zona glomerulosa in rats subjected to stress. These observations suggest that the effect of ACTH on carbohydrate metabolism in the two major zones

of the adrenal cortex may be different. Williams et al (81) propose that in the zona fasciculata the phosphorylase stimulation may be the primary regulatory mechanism, but in the zona glomerulosa the ACTH may act with a different mechanism.

Recently a group of Japanese investigators (83,84) presented evidence that ACTH in vitro increases corticosteroid production by rat adrenal tissue without activation of phosphorylase. In vivo administration of ACTH to rats did not increase adrenal phosphorylase and glucose-6-phosphate dehydrogenase (83, 84). It was shown by Koritz (85) that 3',5'-AMP addition in vitro to rat adrenal glands, stimulated by ACTH, fails to increase further the rate of corticoid production. However, the presence of 3',5'-AMP stimulates further the rat adrenal glands in the presence of saturating concentrations of $NADP^+$ and glucose-6-phosphate. This could not be explained on the basis of increase in $NADPH_2$ as a result of increased breakdown of glycogen. The mechanism of the ACTH action on glucose metabolism and its relation to steroid hormone biosynthesis remains a problem to be solved.

3',5'-AMP and $NADPH_2$ in vitro can overcome inhibition of 11β-hydroxylation by methopyrapone (SU-4885) whereas ACTH can not (86).

The antibiotic puromycin is shown to inhibit protein synthesis in mammalian tissues at the step of

transfer of amino acids from soluble RNA to ribosomal nucleoprotein (87). In vitro puromycin blocks rat adrenal response to ACTH, 3',5'-AMP but does not affect the response to a $NADPH_2$ generating system (88). The inhibitory effect of puromycin on ACTH is reversible. These findings indicate that the steroidogenic effect of ACTH may be mediated through early effect of ACTH on adrenal protein synthesis, and possible activation of enzyme systems involved in steroid hormone biosynthesis.

Hayano and co-workers (89) proposed that ACTH increases the permeability of mitochondria to cholesterol. There is a considerable support for this hypothesis. The enzymes necessary for cholesterol side chain cleavage are localized within the mitochondria (90, 91).

For an optimum effect of ACTH on rat adrenal tissue in vitro calcium and potassium ions as well as glucose are shown to be necessary (92, 93, 94). In rat adrenal homogenates fortified with $NADPH_2$ generating system the calcium ions stimulate the production of Δ^5-pregnenolone from endogenous precursors (95). Freezing of rat adrenal homogenate stimulates corticosteroid formation which is apparently due to increased conversion of cholesterol to 20α-hydroxycholesterol (95). Koritz (96) suggests that this step is rate limiting in the transformation of cholesterol to corticoids in adrenal homogenates.

Recently, McKerns (96) demonstrated that ACTH can stimulate the rate of steroidogenesis up to 7-fold in cell free adrenal preparations in presence of glucose-6-phosphate only. This effect apparently is due to activation of glucose-6-phosphate dehydrogenase in the preparation and an increased generation of $NADPH_2$ necessary for the hydroxylation reactions. Estrogens inhibited the response to ACTH apparently by a competitive inhibition of NADP binding to glucose-6-phosphate dehydrogenase.

The adrenal steroid secretion apart from ACTH is influenced by growth hormone, renin-angiotensin system and some diencephalic factors. These trophic factors are shown to exert their effect mainly on aldosterone secretion.

Growth hormone derived from various species in vitro fails to alter the steroid hormone biosynthesis by rat adrenal glands (26, 34, 35). Administration of growth hormone to hypophysectomized rats increases the adrenal activity for the endogenous biosynthesis of aldosterone in vitro (26, 34). The mechanism of the selective action of growth hormone on rat adrenal activity is not clear. Since the effect is demonstrable only in vivo in hypophysectomized rats there may be several mechanisms involved. Growth hormone may exert directly its effect on the adrenal cortex. In support of this, there

are several studies which show that growth hormone exerts a stimulating effect on adrenal nucleic acid and protein biosynthesis (97).

Growth hormone is shown to have a renotropic effect (98, 99) and it may increase the activity of the renin-angiotensin system, which possesses a stimulating action on the adrenal cortex. Changes in sodium/potassium ratio may be influenced by growth hormone sufficiently to exert a stimulating effect on the zona glomerulosa.

In 1958, Gross (100) directed attention to the renin-angiotensin system as a possible factor for the regulation of mineralocorticoids in vivo. Already in 1951 Deane and Masson (101) observed hypertrophy of the zona glomerulosa with morphological changes indicative of increased secretory activity in rats receiving long term treatment of renin.

Renin is an enzyme produced by the juxtaglomerular apparatus in the kidney. It interacts with plasma alpha-2 globulin fraction which contains renin substrate and splits the leucine-leucine link liberating a decapeptide angiotensin I. The angiotensin I is converted by an activating enzyme to an octapeptide angiotensin II which is the active material responsible for elevation of blood pressure. Angiotensin II is rapidly inactivated by angiotensinase,

present in a variety of tissues, to an inactive amino acid complex. (100).

Several lines of evidence indicate that the renin-angiotensin system is a regulator of aldosterone secretion. Intravenous infusion of both the renin and synthetic angiotensin II increases aldosterone secretion in dogs (102). Microgram quantities of synthetic angiotensin II injected into arterial supply of adrenals of hypophysectomized and nephrectomized dogs increases markedly the secretion of aldosterone (103). Ganong and Mulrow (104) found that low doses of ACTH in dogs produce a prominent effect on cortisol output with less influence on aldosterone, indicating that ACTH and angiotensin stimulate the adrenal cortex in different ways. In nephrectomized hypophysectomized dogs, the increase in aldosterone and 17-hydroxycorticoid secretion produced by infusion of angiotensin II into the arterial blood supplying the adrenal was greater than the increase produced by infusion of the same dose into the jugular vein (103). Similar effect of angiotensin II on transplanted adrenal glands has been shown in sheep by Wright (105), and Blair-West et al (106). It was pointed out earlier that in normal rats angiotensin II and crude kidney extract fails to increase the secretion of aldosterone. However in hypophysectomized animals

angiotensin II increases aldosterone secretion with little change in corticosterone (32). In vitro incubation of rat adrenal glands with angiotensin II has failed to show a significant increase in aldosterone secretion (107). Beef adrenal glands in vitro respond to angiotensin II in a similar way as with ACTH (107).

Angiotensin II stimulates aldosterone secretion with exogenous cholesterol but has no effect with exogenous progesterone or corticosterone (107). Glaz and Sugar (33) treated normal rats with angiotensin II injections and showed that the adrenal glands from these animals on incubation in vitro had a higher capacity for aldosterone production. Studies with synthetic angiotensin II - amide by Marx et al (108) on subcutaneous administration to normal male rats show that this angiotensin derivative caused histological and histochemical alterations in the cells of the adrenal zona glomerulosa indicative of increased secretory activity. Histochemical reaction for Δ^5-3β -hydroxysteroid dehydrogenase was increased; also, glucose-6-phosphate dehydrogenase activity was augmented. The inner zones of the adrenal cortex showed no demonstrable changes after angiotensin amide treatment. Adrenal glands from angiotensin treated animals in vitro secrete higher amounts of aldosterone and 18-OH-B in the presence of ACTH than do adrenals from the untreated controls. Carr and Bartter (109) compared the action of angiotensin II

and ACTH on rat adrenal ascorbic acid. In hypophysectomized rat ACTH depletes adrenal ascorbic acid whereas angiotensin II does not. The authors suggest that this test can be used to detect ACTH in biological fluids or extracts which stimulate steroidogenesis by the adrenal cortex.

In 1956, Rauschkolb and Farrell (110) advanced a hypothesis that aldosterone secretion is regulated by a circulating hormone elaborated by the diencephalon. Farrell and co-workers (111, 112) have observed stimulation of aldosterone secretion by extracts of diencephalon and the pineal gland in decerebrated dogs. A substance, 1-methyl-6-methoxy-1,2,3,4-tetrahydro-2-carboline has been isolated from pineal extracts which was suspected of being the active principle involved in stimulation of the secretion of aldosterone (113). This substance, according to Farrell (106), does not account for the aldosterone stimulating action present in diencephalon extracts. In vitro fresh rat pineal tissue and various extracts of the pineal gland have failed to increase aldosterone secretion by the adrenal glands of this species (14, 26). Fresh hog diencephalon homogenate and some commercial crude posterior pituitary extracts exert a stimulating action on aldosterone biosynthesis of rat adrenal quarters in vitro (14, 26). The nature of the aldosterone-stimulating material occasionally demonstrable in midbrain extracts remains unknown.

II. METHODS AND MATERIALS

1. Paper chromatography of steroids:

First attempts at separation of coloured substances by paper chromatography were made by Runge (114) some 100 years ago. Little attention was paid to these observations, until 1944 when Consden, Gordon and Martin (115) introduced paper chromatography in the separation of amino acids. The separation of the sterols and steroids by this procedure was made feasible to paper partition chromatography by the introduction of selected solvent systems by Zaffaroni and Burton (116) in 1951 and by Bush (117) in 1952. These authors established two different systems for the separation of steroids.

The Zaffaroni systems call for the impregnation of the paper with the stationary phase prior to the application of extracts, the mobile phase being an organic solvent previously saturated with the corresponding stationary phase.

The following systems were used in the present study:

1. T.E.G.	toluene/ethylene glycol	(118)
2. T.P.G.	toluene/propylene glycol	(119)
3. L.P.G.	ligroin/propylene glycol	(120)
4. B.F.	benzene/formamide	(119)

5. $CHCl_3F$. chloroform/formamide (119)
6. B.E.F. buthylacetate:ethylacetate/water: formamide (85:15/5) (121)

The systems developed by Bush are aqueous multi-solvent systems, where the stationary phase is contained in the atmosphere of the chromatography tank. Extracts for separation are applied on dry paper strips. The chromatogram is equilibrated in the chromatography tank for 30 minutes at 25°C before addition of the mobile phase.

The following Bush systems were used:

1. Bush A petroleum ether/methanol:water (100/80:20) (117)
2. Bush B_5 benzene/methanol: water (100/50:50) (117)
3. Bush C toluene:ethylacetate/methanol:water (90:10/50:50) (117)
4. $B.L_1$ petroleum ether:benzene/methanol: water (30:70/50:50) (122)
5. D. methylcyclohexane:toluene/methanol: water (100:25/80:20) (123)
6. E_2B. isooctane/t-butanol:water (100/50:90) (124)
7. E_4 isooctane/t-butanol:methanol:water (100/45:45:10) (124)

a. Preparation of paper for chromatography: Whatman No. 1 chromatography paper was employed for all separations.

The paper, prior to chromatography, was continuously extracted in a Soxlet apparatus with methanol and benzene for 3 days to remove soluble contaminants which interfere with microchemical colour reactions. After extraction the paper strips were dried and stored in a dust free folder.

b. Chromatography of extracts: Washed paper, 17 cm wide and 56 cm long, was cut into limbs from 1.0 to 10 cm wide with a common head 15 cm long. Each limb is separated by 1 cm wide cut out space, to prevent the lateral contact between the strips. The cutouts extend into the common head 1.5 to 2 cm above the starting line to avoid cross-contamination of the extracts between the adjacent limbs.

Extracts for chromatography were dissolved with a few drops of acetone and applied by means of a capillary pipette on the starting line. The solvent was evaporated by a stream of nitrogen. To secure a quantitative application the tube containing the extract was washed 3 times with 5 drops of acetone. The prepared chromatograms were suspended in chromatography tanks and developed by a descending technique. All chromatograms contained at least one pilot strip with appropriate reference substances and dyes.

c. Running rates of steroids: After development of the chromatogram, the paper was dried overnight and the steroid zones were located by means of ultraviolet light

(Mineral light λ max. 240-250 mμ) or microchemical test reactions. Running rates in systems which have no runoff are expressed as 'R_F'

$$R_F = \frac{\text{centre of steroid zone in cm from origin}}{\text{cm of solvent front from origin}}$$

In systems where the solvent front runs off the paper the relative running rate is determined with respect to a known reference substance (R_S) e.g. R_{DOC}

$$R_{DOC} = \frac{\text{cm of unknown from origin}}{\text{cm of deoxycorticosterone from origin}}$$

In the case of R_F the value is smaller than 1.0 or equal to 1.0, whereas for R_S the values may be smaller - equal or larger than 1.0.

Both methods for calculation of running rates have been used in the present study.

d. Systematic separation of steroid hormones: A combination of the Zaffaroni and Bush type of chromatographic solvent systems has been used to separate the steroid hormones extractable from adrenal incubates. The extract was applied and chromatographed in the T.E.G. system for 72 hours. On the pilot strip 4 different dyes were used as visual indicators:

F_{14} (blue) after 72 hours remains in lower third

F_5 (red) runs off after 24 hours

F_9 (purple) runs off after 8 hours.

Sudan red runs off after 5 hours.

Three different overflows were collected from the original paper strip.

1. The overflow collected during the first 6 hours contained compounds of low polarity. The following steroid standards appear in this fraction: 6β-OH-prog., 11β-OH-prog., 17α-OH-prog., 18-OH-prog., testosterone, epitestosterone, 19-nortestosterone, DOC, 11-keto-prog., androstenedione, progesterone, 16-dehydro-prog.

2. The overflow collected from 6 - 24 hours contained the following steroid standards: 18-OH-DOC, 6β-OH-DOC, B, S, 16α-OH-prog., A, 11β-OH-androstenedione, dihydro-A.

3. The overflow collected from 24 to 72 hours contained 19-OH-DOC.

On the original paper remained the following reference standards: 6β-OH-F, 6β-OH-E, 16α-OH-B, 18-OH-B, 6β-OH-S, 19-OH-B, cortisol, aldosterone.

2. Location of steroid hormones on chromatograms:

Steroids containing conjugated double bonds (Δ^4-3 keto structure) absorb ultraviolet light in the region of 240 mμ. On exposure of the chromatogram to ultraviolet light source using a Corning filter no. 9863, such steroids appear as dark zones. The sensitivity for visual detection

is in the region of 2 $\mu g/cm^2$. For permanent records, the chromatograms were photographed with ultraviolet light using Kodak standard contact photographic paper. This method of steroid detection is most useful since the steroid on the paper does not suffer a demonstrable destruction.

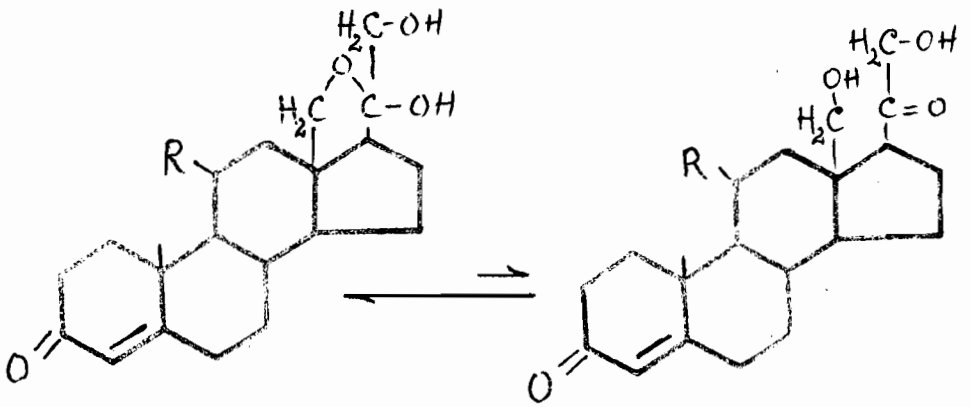
Microchemical test reactions on the paper are rather sensitive and relatively specific but involve destruction of the original compound. These reactions have been applied on a narrow (2 - 5 mm wide) strip cut out from the original chromatogram.

The following test reactions which are specific for various reactive groups of the steroid molecule have been used:-

Aqueous sodium hydroxide reacts with Δ^4-3 keto structure. The test strip is dipped into 2N NaOH solution and subsequently dried at 90°C for 20 minutes (125). Most of the Δ^4-3 keto steroids give a bright yellow fluorescence when exposed to ultraviolet light (350 mμ). The sensitivity of this method for aldosterone, corticosterone, cortisol is as low as 0.1 $\mu g/cm^2$. Steroid acetates also give this reaction. There are several Δ^4-3 keto steroids which do not give the soda fluorescence reaction. 7α-OH-DOC does not show fluorescence and 19 hydroxylated steroids

such as 19-OH-DOC, 17α,19-OH-DOC and 19-OH-DOC acetate yield blue-green fluorescence characteristic for this group of compounds. Yellow greenish soda fluorescence is obtained with 19-OH-B (126). The soda fluorescence method is usually combined with the alkaline blue tetrazolium (BT) reaction.

Steroids with an α-ketol structure reduce alkaline BT solution forming a blue formazan zone (125). The chromatogram is drawn through a fresh mixture of 1 volume of 0.1% BT+ 9 volumes 2N NaOH and laid on a glass plate. An immediate reaction takes place with most C_{21} steroids possessing 20-keto,21 hydroxy groups and with C_{18} steroid α-ketols which have 17 keto,16 hydroxy or 16 keto,17hydroxy groups. The colour development is markedly hindered for 18-OH-B and 18-OH-DOC. These compounds exist predominantly in the 18 → 20 cyclohemiketal form. An opening of the cyclohemiketal ring takes place very slowly which explains the delayed blue formazan formation. Tautomeric equilibrium of this hemiketal structure is shown below:



BT negative BT positive

R = -H (18-OH-DOC)

R = -OH (18-OH-B)

The 18 monoacetate of 18-OH-B has an open structure and it reacts quickly with the BT reagent. As the acetate group is split off, a cyclic 18 $\rightarrow$ 20 hemiketal forms and slows down the color development (127). In the case of aldosterone the cyclic hemiacetal forms between C-18 and C-11 which does not interfere with the α-ketol structure and the BT color development. Some steroids with a glycol side chain give a weak, slowly-developing formazan color. An interpretation of the BT reaction was made taking into consideration the rapidity of the color development, by observing the test strip for 30 minutes. For most C_{21} steroids with an α-ketol side chain the sensitivity of the BT reaction is in the region of 0.2 $\mu g/cm^2$.

The 17 ketosteroids are detected by means of the Zimmerman reaction. The method described by Savard (120) has been slightly modified. The chromatogram is immersed in aqueous 5N KOH solution, and excess reagent is removed by blotting between sheets of filter paper. The strip is drawn through a 2% solution of meta-dinitrobenzene in ethanol and blotted again. On gentle warming at 65°C for 10 minutes an intense violet color develops in areas where 17 ketosteroids are present.

Steroids with a 3-keto group, give a less intense blue-violet color and those with a 20-keto group, a brown-purple color. The limit for detection of 17 ketosteroids is about 2 to 5 $\mu g/cm^2$.

The phosphoric acid reaction described by Neher and Wettstein (128) gives different fluorescence colors with various corticosteroids. A strip of the chromatogram is drawn through a 15% aqueous phosphoric acid, blotted and dried at 90°C for 20 minutes to complete dryness. The fluorescent steroid zones are located by a long wave ultraviolet light source (350 mμ).

Steroids with Δ^5-3β -OH are detected with a sulfuric acid reagent. The chromatogram is drawn through a mixture containing 3 parts concentrated sulfuric acid and 1 part 90% ethanol, and laid on a glass plate. A bright yellow color indicates the location of the Δ^5-3β OH steroid. Sensitivity of detection is in the order of 5 $\mu g/cm^2$.

Phenolic steroids are located on the chromatograms by the Folin-Ciocalteau reagent (129). The paper strip is sprayed with diluted reagent (1 part Folin-Ciocalteau reagent prepared by the British Drug Houses Ltd., and 4 parts distilled water). On exposure of the chromatogram to ammonia vapor, the areas containing phenolic steroids assume a blue color. The intensity of the color varies with the nature of the steroid. Estradiol-17β and estradiol-

17α give the weakest color. The sensitivity is in the range from 5 to 10 $\mu g/cm^2$. Ortho dihydroxy estrogens: 2-hydroxy-estradiol-17β and 2-hydroxy-estrone react immediately with Folin-Ciocalteau reagent without exposure to ammonia vapors.

3. Thin layer chromatography:

The first attempts to use thin layer chromatography (TLC) were described by Izmailov and Schraiber over 20 years ago (130). This method was further developed by Stahl (129), who described a practical method for coating glass plates and demonstrated the usefulness of TLC for separation of a wide range of substances. Separation of steroid hormones on TLC depends on the differences in the adsorption properties of the hormones, the adsorbent layer used and on the polarity of the developing solvent. For the final separation of some of the isolated steroids TLC. has been applied. The adsorbent, Silica Gel H (Stahl) is coated on a glass plate to a thickness of 0.25 mm in the form of aqueous slurry. Coated plates are dried and activated at 110oC for 30 minutes and stored in a desiccator.

The extract is applied in the form of a spot and the plate is developed by the ascending technique in a chromatography tank, allowing the solvent front to travel for 15 cm above the spot of application. The hormones are

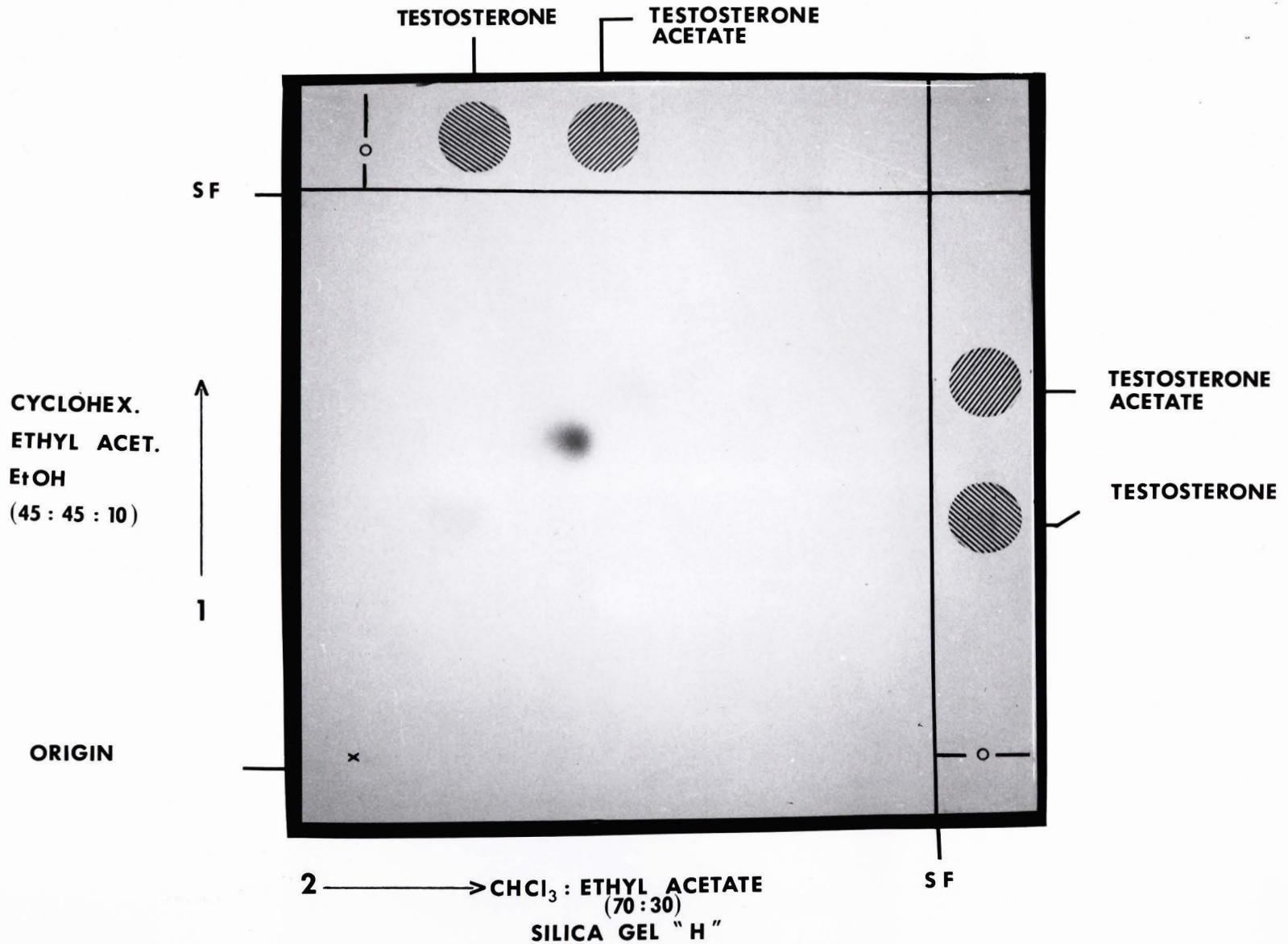
detected on the TLC plate by the same methods as described for paper chromatograms. TLC offers an advantage in the use of corrosive reagents for the detection of separated steroids.

A radioautogram of a two dimensional TLC of the "testosterone acetate" zone isolated from rat adrenal glands is shown in Figure 1. The radioactivity separates from the authentic carrier thus disproving the identity.

4. Detection of radioactivity on the chromatograms:

Radioactive conversion products derived from ^{14}C-labelled precursors are detected by radioautography. The chromatogram is covered with a no-screen Kodak high sensitivity X-ray film in a light-tight standard X-ray cassette. On exposure of the chromatogram to the film for 3 to 5 days the β radiation of ^{14}C reduces the photographic emulsion. The films after exposure are developed by standard X-ray film developing techniques. The radioactivity of 2000 - 3000 dpm/cm^2 of ^{14}C (absolute disintegrations per minute) can be detected on the film after an exposure for 3 days. The radioautograms are useful for the detection of the outline of the radioactive zone present on the chromatogram, which allows a precise cutting out of the compound for elution. The intensity of the radioactive zones on the film are used also as a guide for the size of aliquots

Figure 1. Acetylation of conversion product in the 17α -OH-prog.-testosterone area; its non-identity to authentic testosterone acetate in two dimensional TLC.


TESTOSTERONE
TESTOSTERONE ACETATE
SF
CYCLOHEX.
ETHYL ACET.
EtOH
(45 : 45 : 10)
1
ORIGIN
TESTOSTERONE ACETATE
TESTOSTERONE
2 → CHCl3 : ETHYL ACETATE (70 : 30)
SILICA GEL " H "
SF

which should be taken for assay in the liquid scintillation spectrometer. Radioautograms are permanent records for purposes of comparison of different experiments.

5. Assay of radioactivity:

Radioactivity of ^{14}C labelled compounds was assayed in a Packard liquid scintillation spectrometer using 5 ml of toluene phosphor containing 0.3% of 2,5-diphenyloxazole (POP) and 0.01% p-bis 1,2(5 phenyloxazolyl) 1-benzene (POPOP). The efficiency for ^{14}C in this system has been estimated to be 60%. Aliquots of 1/100 to 1/10 of the total sample were used for assay of the radioactivity. These aliquots were completely dried in the counting vials under nitrogen and redissolved in the scintillation phosphor. The assay of radioactivity can be done with an accuracy of $\pm 2\%$.

6. Quantitative determination of steroid hormones:

a. Ultraviolet light absorption method: Steroid hormones with a Δ^4-3 keto structure absorb ultraviolet light in the region of 240 mμ. The steroid is dissolved in ketone free ethanol and the ultraviolet light absorption spectrum is obtained in a Beckman DU spectrophotometer using silica cells. The quantity of the steroid hormone present in the solution is calculated using the

Beer-Lambert's law according to the following formula:-

$$\mu g \Delta^4\text{-3 ketosteroid} = \frac{OD\ max \times MW \times V \times 1000}{E}$$

OD max = maximal optical density

MW = molecular weight of the steroid

V = volume in milliliters

E = molar extinction coefficient of the steroid.

b. Blue tetrazolium (BT) reaction: Steroid hormones with an α-ketol side chain were measured quantitatively by the BT reaction described by Nowaczynski et al (131).

c. Isonicotinic acid hydrazide reaction (INH): The 3 keto group of Δ^4-3 keto steroids in acid alcohol reacts with isonicotinic acid hydrazide forming yellow hydrazone (132). This reaction proceeds at room temperature and is complete after 60 minutes (26). The INH reagent contains 25 mg% isonicotinic acid in acid ethanol (0.625 ml HCl/liter ethanol). To the dried steroid residues ranging from 2 to 14 µg are added 3 ml INH reagent solution. The samples are gently shaken in stoppered tubes and allowed to stand in the dark for 1 hour. The developed yellow colour is measured in a Beckman DU spectrophotometer in silica cells at wave length 380 mµ. The readings of steroids eluted from chromatograms are corrected by subtracting a corresponding paper blank and the blank of the

reagent. Sensitivity of this method is as low as 2 µg of Δ^4-3 keto steroid.

7. Preparation of derivatives of steroid hormones:

a. Acetylation: Isolated steroid hormones combined with authentic carrier have been acetylated (133). The dry steroid residue was dissolved in 0.1 ml of dry pyridine and 0.1 ml of acetic anhydride was added to the solution. The tubes were stoppered and incubated at room temperature for 18 hours. In some instances, 0.1 ml of dry pyridine and 0.4 ml of acetic anhydride has been used for acetylation at 60°C for 15 minutes. After the acetylation reaction was completed, the reagents were evaporated at room temperature under a stream of nitrogen. Both methods acetylate the steroid hydroxyl groups at the positions 21 and 18. The hydroxyl groups at C-17 and C-11 are not affected. In case of 18-OH-B a mixture of 21 acetate and 18,21 diacetate was obtained.

b. Hydrolysis: Steroid acetates were hydrolysed using a method described by Bush (134) with minor modifications. The steroid was dissolved in 0.5 ml methanol and 0.5 ml of 2.5% sodium carbonate was added. The solution was kept for 30 minutes at room temperature and extracted with ethyl acetate.

c. Oxidation: Sodium bismuthate method has been used for the oxidation of C_{21} steroids containing the 17α-

hydroxy group to 17 ketosteroids (135). The steroid was dissolved in 0.5 ml of glacial acetic acid to which were added 0.5 ml of water and 25 mg of sodium bismuthate. The preparation was kept for 2 hours in the dark at room temperature and subsequently extracted with ethyl acetate. The ethyl acetate extract was washed with saturated sodium carbonate and finally with water, then dried and evaporated.

Oxidation of 11-hydroxy group to 11-keto group was accomplished with the chromic acid method (136). The steroid was dissolved in 0.5 ml of glacial acetic acid to which 0.6 ml of 0.2% chromic acid solution was added. The preparation was kept at room temperature in the dark for 20 minutes, then diluted with 5 ml of water and extracted using ethyl acetate. The ethyl acetate was washed with saturated sodium carbonate and subsequently with water, then dried and evaporated.

8. Incubation of rat adrenal glands:

For this study, adrenal glands from hooded male rats (body weight 150 - 200 gm) were used. In some instances adrenal glands from hypophysectomized male rats (150 - 180 gm) were also incubated. For adrenalectomy the animals were anaesthetized with sodium pentobarbital 12 - 18 mg intraperitoneally. The adrenal glands were dissected from surrounding fat tissue, quartered and weighed. Adrenal tissue

preparations were kept in Krebs Ringer bicarbonate buffer cooled over ice until the time of incubation.

As the incubation medium Krebs Ringer bicarbonate buffer pH 7.4 (137) with 200 mg% of glucose (KRG) has been used. The incubation was carried out in a Dubnoff metabolic shaking incubator at 37.5°C under 95% O_2 and 5% CO_2 for variable time intervals. At the end of the incubation, the medium was decanted, and the tissue washed 3 times with 5 ml of fresh KRG solution. These were combined and extracted 3 times with 50 ml portions of ethyl acetate. The combined ethyl acetate extracts were dried over sodium sulfate, filtered and evaporated.

In order to determine whether steroids remain in the gland, the tissue was homogenized with 5 ml KRG solution and extracted with 4 volumes of distilled acetone overnight at 4°C. The precipitate was filtered off and washed 3 times with 10 ml of fresh acetone. The acetone extract was combined with the washings and was evaporated in vacuo at 35°C until an aqueous residue was obtained. The aqueous residue was then re-extracted 3 times with 50 ml portions of distilled ethyl acetate. The ethyl acetate extracts were combined and dried over anhydrous sodium sulfate, then separated from sodium sulfate and evaporated to dryness in vacuo at 50°C. The extracts

from the incubation medium and the tissue were then subjected to chromatographic fractionation in the systems described.

9. Labelled steroid hormone precursors:

Labelled progesterone-4-^{14}C (sp. act. 1 µc/14.3 µg and 1 µc/12.0 µg) and 11-deoxycorticosterone-4-^{14}C (sp. act. 1 µc/9.4 µg) obtained from New England Nuclear Corp., have been used. Prior to the incubation experiments these radioactive hormones were purified by paper chromatography. Aliquots of 1 to 2 µc were measured into incubation vessels, evaporated under nitrogen and redissolved into 3 drops of propylene glycol. To these preparations were added KRG buffer and adrenal tissue according to the plan of the experiment.

III. RESULTS

1. Studies with the Mattox modification of the Porter-Silber reaction:

The Porter-Silber reaction is regarded as specific for steroids with a dihydroxy acetone side chain (138).

Lewbart and Mattox (139) demonstrated that the C-17 side chain of corticosterone and aldosterone could be transformed to a glyoxal structure with cupric acetate. The modified steroid reacts with the Porter-Silber reagent yielding a yellow chromogen. This method has been studied for its applicability to the quantitative assay of corticosterone, aldosterone, 18-OH-B and 18-OH-DOC.

Reagents:

1. Cupric acetate solution - 0.005M cupric acetate in methanol.
2. Porter-Silber reagent consists of phenylhydrazine (0.065 gm) dissolved in 100 ml of 62% sulfuric acid. The reagent is prepared just before use and can not be stored.

Procedure:

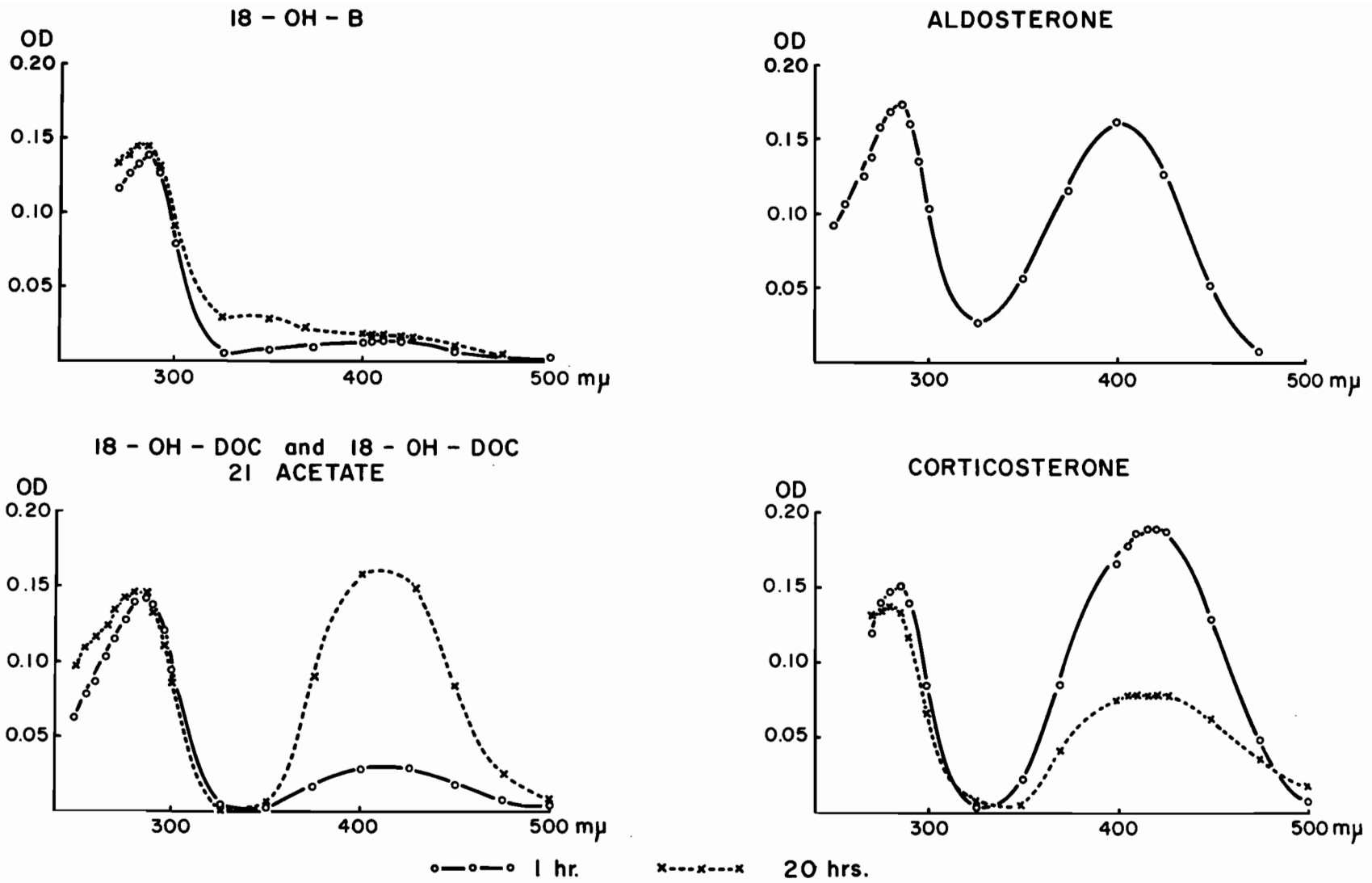
To dry steroid residues is added 0.1 ml of cupric acetate solution; after 30-60 minutes at room temperature, the solvent is evaporated under a stream of nitrogen to dryness. The Porter-Silber reagent (3.0 ml) is added to

the tubes which are then stoppered, gently shaken and kept at room temperature for 1 to 20 hours. With each set of test samples a reagent blank is prepared. The spectra of light absorption in the samples are read in a Beckman DU spectrophotometer using silica cells with 1 cm light path.

Aldosterone and corticosterone form rapidly a yellow color with the reagent which is maximally developed after 1 hour at room temperature. The color formation of 18-OH-DOC and 18-OH-DOC 21 acetate is slow and requires 20 hours. No visible color in the Mattox Porter-Silber reaction is observed with 18-OH-B.

As shown in Figure 2, the spectra of aldosterone, corticosterone, 18-OH-DOC and 18-OH-DOC 21 acetate after the Mattox Porter-Silber reaction exhibit 2 absorption maxima: one in the region of 280-285 mμ and the other at 400-420 mμ with a minimum at 325 mμ. The first light absorption maximum (280-285 mμ) is formed within one hour of reaction and for 18-OH-DOC and corticosterone does not change appreciably after 20 hours of reaction. The second maximum at 420 mμ of corticosterone diminishes on standing at room temperature. A reverse effect takes place with 18-OH-DOC and 18-OH-DOC 21 acetate, where the light absorption maximum at 415 mμ increases after 20

Figure 2. Spectra of Mattox Porter-Silber chromogens.


18 - OH - B
OD
0.20
0.15
0.10
0.05
300
400
500 mμ
ALDOSTERONE
OD
0.20
0.15
0.10
0.05
300
400
500 mμ
18 - OH - DOC and 18 - OH - DOC
21 ACETATE
OD
0.20
0.15
0.10
0.05
300
400
500 mμ
CORTICOSTERONE
OD
0.20
0.15
0.10
0.05
300
400
500 mμ
1 hr.
20 hrs.

hours of reaction. The reason for this effect is not clear and the chemical changes in the configuration of this compound in the Mattox Porter-Silber reaction has not been studied.

Only one distinct maximum at 285 mμ is formed by 18-OH-B which appears after 1 hour and remains unchanged after 20 hours.

The optical density of the light absorption maxima formed in the Mattox Porter-Silber reaction is linearly proportional to the amount of the steroid in the range from 2 - 10 μg. This reaction offers an advantage for the quantitative assay of 18-OH-DOC, aldosterone and corticosterone where two quantitative readings can be obtained with one sample. The eluted paper blanks in the Mattox Porter-Silber reaction are low. For quantitative assays the optical density readings are taken at 285 mμ and 415 mμ. Aldosterone and corticosterone are measured after 1 hour and 18-OH-DOC after 20 hours. 18-OH-B is measured at 285 mμ after 1 hour.

2. Chromatographic mobilities of steroid hormones:

Steroid hormones from C_{21}, C_{19} and C_{18} series as well as various steroid acetates have been chromatographed in order to establish the positions of these hormones in various solvent systems. The positions of the steroid hormone zones on the chromatograms were detected by the

methods previously described. Results are compiled in Tables I - IX. Several exceptions in the various test reactions have been noted.

The soda fluorescence reaction does not take place with 19-aldo-androstenedione, progesterone, 16α-OH-prog., 6β-OH-prog., 20β-hydroxy,pregn-4-ene-3-one, 17α-OH-prog., and 16-dehydro-prog. Other derivatives of progesterone give a soda fluorescence reaction with variable colors: 11β-OH-prog. - ochre, 18-OH-prog. - deep yellow, 11-keto-prog. - pinkish-yellow. A blue-green fluorescence is obtained with 19-OH-DOC and 19-OH-DOC acetate. A yellow-greenish fluorescence was produced by 19-OH-B and a blue-pink fluorescence was obtained with 19-OH-testosterone. These findings indicate that the soda fluorescence reaction is influenced by certain structural groups of the steroid molecule. Derivatives of progesterone become fluorescent if oxygenated at C-11 or C-18 but do not exhibit a typical yellow fluorescence similar to that produced by DOC. Androstenedione gives a typical yellow soda fluorescence whereas 19-aldo-androstenedione does not give this reaction.

The BT reaction on paper with 18-OH-B and 18-OH-DOC showed a delayed and faint purple formazan formation.

TABLE I

R_s VALUES (S = CORTISOL) of C_{21} STEROIDS

Steroid	Chromatographic Solvent Systems								
	T.E.G.	B.F.	$CHCl_3F$.	Bu.B5	Bu.C	B.L1	D.	E_2B.	B.E.F.
6 β-OH-F	0.02			0.03	0.05				
6 β-OH-E	0.09			0.10	0.13				
16α-OH-B	0.25			0.19	0.21				
18-OH-B	0.53	0.42	0.90	0.46	0.36	0.44			0.63
6 β-OH-S	0.53			0.53	0.58	0.45			
19-OH-B	0.67								0.61
Cortisol(F)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Aldosterone	2.32	1.28	2.82	1.54	1.03	1.59	2.23	0.77	0.54
Cortisone(E)	2.94	1.95	2.94	1.90	1.56	2.13	2.54	1.88	
19-OH-DOC			3.50		1.55				0.86
18-OH-DOC	8.4			2.80	1.96	4.85	9.77		
6 β-OH-DOC		6.11			1.96	4.41	6.73		
Corticosterone (B)			4.74	3.25	2.12	6.60			1.11

TABLE LI

R_S VALUES (S = CORTICOSTERONE) of C_{21} STEROIDS

Steroid	Chromatographic Solvent Systems									
	T.E.G.	L.P.G.	T.P.G.	B.F.	$CHCl_3F$	BuB_5	Bu.C	B.L$_1$	D.	E_2B
18-OH-B					0.19	0.15		0.05		
Cortisol(F)					0.21	0.33	0.47			
Aldosterone					0.59	0.45	0.48	0.24		
Cortisone(E)				0.15	0.62	0.57	0.74		0.19	
19-OH-DOC	0.30				0.74	0.67	0.73			
18-OH-DOC	0.49			0.45		0.88	0.92	0.74	0.91	1.05
6 β-OH-DOC	0.52	0.63	0.60	0.40			0.92		0.63	0.82
11-deoxy-cortisol(S)	0.77	1.00	0.76	0.90					1.15	1.23
Corticosterone(B)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
16 α-OH-prog.	1.41	2.50	1.47	1.73					1.51	1.26
11-dehydro-corticosterone(A)	1.80	3.38	2.41	1.79		1.09			1.36	0.80

(continued)

TABLE II (continued)

R_S VALUES (S = CORTICOSTERONE) of C_{21} STEROIDS

Steroid	Chromatographic Solvent Systems									
	T.E.G.	L.P.G.	T.P.G.	B.F.	$CHCl_3F$.	BuB5	Bu.C	B.L1	D.	E_2B.
Dihydro-A	2.13	0.21		2.65						1.08
THA				1.05						
6 β-OH-prog.									4.73	1.21
17α-OH-prog.										1.48
18-OH-prog.										1.45
11-deoxy-corticosterone(DOC)						1.25				1.45
Progesterone						1.25				
17α-OH-pregnenolone										1.60

4766.

TABLE III

R_S VALUES (S = DOC) of C_{21} STEROIDS

Steroids	Chromatographic Solvent Systems					
	T.E.G.	L.P.G.	T.P.G.	Bu.B_5	Bu.A	D.
18-OH-B				0.12		
Aldosterone				0.36		0.04
19-OH-DOC			0.06	0.54		
Corticosterone(B)				0.80		0.10
16α-OH-prog.	0.68	0.07				0.24
11-dehydro-corticosterone (A)	0.68			0.88		0.15
Dihydro-A						0.30
6β-OH-prog.	0.87	0.21			0.28	0.58
11β-OH-prog.	0.97	0.30			0.28	0.67
17α-OH-prog.	0.97	0.48	0.82		0.50	0.86
18-OH-prog.	0.97	0.64				2.05

(continued)

TABLE III (continued)

RS VALUES (S = DOC) of C_{21} STEROIDS

Steroids	Chromatographic Solvent Systems					
	T.E.G.	L.P.G.	T.P.G.	Bu.B5	Bu.A	D.
11-deoxy-corticosterone (DOC)	1.00	1.00	1.00	1.00	1.00	1.00
20 β-OH-pregn-4-ene-3,20-dione	1.02				1.79	
11-keto-prog.	1.05	1.80			1.00	1.10
Pregnenolone		2.23				
Progesterone	1.05	6.67		1.00	2.72	2.14
16-dehydro-prog.		8.43				

TABLE IV

TEST REACTIONS OF C_{21} STEROIDS

Steroid	NaOH Fl.	BT	H_3PO_4
6 β-OH-F	+	+	blue
6 β-OH-E	+	+	bluish
16α-OH-B	+	+	yellow-green
18-OH-B	+	-*	-
6 β-OH-S	+	+	blue-purple
19-OH-B	yellow-greenish	+	blue
Cortisol (F)	+	+	yellow-green
Aldosterone	+	+	-
Cortisone (E)	+	+	white-bluish
19-OH-DOC	blue-green	+	-
18-OH-DOC	+	-*	-
6 β-OH-DOC	+	+	-
11-deoxy-cortisol(S)	+	+	orange-reddish
Corticosterone(B)	+	+	yellow-green
16α-OH-prog.	-	-	-
11-dehydro-corticosterone (A)	+	+	faint blue
Dihydro-A	-	+	
THA	-	+	

(continued)

TABLE IV (continued)

TEST REACTIONS OF C_{21} STEROIDS

Steroid	NaOH Fl.	BT	H_3PO_4
6 β-OH-prog.	-	-	-
11 β-OH-prog.	ochre	-	yellowish
17 α-OH-prog	-	-	-
18-OH-prog.	deep yellow	-	-
DOC	+	+	orange
20 β-hydroxy,pregn-4-ene,3-one	-		
11-keto-prog.	pinkish-yellow	-*	-
Progesterone	-	-*	yellow
16-dehydro-prog.	-	-	-

\+ positive (for NaOH Fl. - typical yellow)
(for BT - rapid blue color formation)

\- negative

-* delayed faint color

TABLE V.

R_S VALUES (S = CORTISOL) of C_{19} STEROIDS

Steroid	Chromatographic Solvent Systems	
	T.E.G.	Bu.C
19-OH-testosterone	1.97	1.15
19-OH-androstenedione		2.11
19-aldo-androstenedione		3.05

TABLE VI

R_S VALUES (S = CORTICOSTERONE) of C_{19} STEROIDS

Steroid	Chromatographic Solvent Systems						
	T.E.G.	L.P.G.	T.P.G.	B.F.	Bu.C	D.	E_2B.
19-OH-testosterone	0.10		0.12		0.49		
19-OH-androstenedione	0.96		0.90	1.00	0.93		0.96
3β,11β-dihydroxy-5α-androstane-17-one							0.14
3β,17β-dihydroxy-androst-5-ene							1.82
DHEA	3.49		3.50				1.57
11β-OH-androstenedione	2.47	4.15	2.22	3.11		3.46	1.26
Testosterone	2.53		2.70				1.53
Adrenosterone	3.59		4.88			5.90	1.09
19-aldo-androstenedione	3.52		4.91		1.26		
Androstenedione	3.96		5.40				

TABLE VII

R_S VALUES (S = DOC) OF C_{19} STEROIDS

Steroid	Chromatographic Solvent Systems				
	T.E.G.	L.P.G.	T.P.G.	Bu.A	D.
19-OH-androstenedione	0.28	0.03			
DHEA		1.15			
11 β-OH-androstenedione		0.12			0.31
Testosterone		0.64	0.67	0.96	0.83
Adrenosterone	1.01	0.90	1.10	0.45	0.64
Epitestosterone		0.91			
19-aldo-androstenedione	1.05	1.33		0.87	
Etiocholanolone		1.50			
Androsterone		2.90			
Androstenedione	1.08	3.21		2.00	1.65

TABLE VII a

TEST REACTIONS OF C_{19} STEROIDS

Steroid	NaOH Fl.	D.N.B.	H_2SO_4	H_3PO_4
19-OH-testosterone	blue-pink	neg.		yellow
19-OH-androstenedione		+		neg.
3β,11β-dihydroxy-5α-androstane-17-one		++	neg.	
3β,17β-dihydroxy-androst-5-ene			pos.	
DHEA		++	pos.	purple
11β-OH-androstenedione	yellow	++		yellow-orange
Testosterone	yellow	+++		faint-yellow
Adrenosterone	yellow	+++		neg.
Epitestosterone		neg.		
19-aldo-androstenedione	neg.	+		faint purple
Etiocholanolone		+++	neg.	
Androsterone		+++	neg.	
Androstenedione	yellow	+++		neg.

D.N.B. (Zimmermann reaction)

\+ pale color

++ moderate color

+++ strong color.

TABLE VIII

R_S VALUES OF C_{18} STEROIDS

Steroid	Chromatographic Solvent Systems					Remarks
	T.E.G. R_S=F	Bu.C R_S=F	T.E.G. R_S=E	E_2B. R_S=B	LPG R_S=DOC	
Estriol	0.01					
2-OH-estradiol-17β	0.35					UV+, Folin-Ciocalteau + without NH_4OH
16-epi-estriol	0.61					tailing
16-keto-estrone	2.03	2.73				
Paraquinol		1.80				UV +, Folin-Ciocalteau -
16-keto-estradiol-17β	3.55	2.41	1.32			
16α-OH-estrone		2.54				
Estradiol-16α			2.27			
Estradiol-16β			2.73			
Estradiol-17β		2.73	2.73			
Estradiol-17α			3.19			
Estrone-16-keto				1.63		
Estrone				1.63		
OTHER COMPOUNDS						
19-nortestosterone				1.45	0.45	UV +, Folin-Ciocalteau -
19-norandrostenedione					3.27	UV +, Folin-Ciocalteau -
Bisdehydrodoisynolic acid	0.80					
Aromatized prog.					0.26	tailing

TABLE IX

R_F VALUES OF STEROIDS AND THEIR ACETATES

C_{21} Steroids	Chromatographic Solvent Systems						
	T.P.G.	B.F.	BL1	D.	E_2B.	E_4	Bu.A
18-OH-B	0.01		0.02			0.54	
18-OH-B 21 acetate	0.12		0.34			0.77	
18-OH-B 18,21 diacetate	0.48		0.69			0.82	
19-OH-B	0.01		0.04				
19-OH-B 19,21 diacetate	0.57		0.8				
Cortisol (F)	0.01		0.06				
Cortisol 21 acetate	0.1		0.52	0.04		0.82	
Aldosterone			0.13	0.02			
Aldosterone acetate			0.73	0.05			
Cortisone (E)	0.03		0.13				
Cortisone 21 acetate	0.21		0.63	0.06			
19-OH-DOC	0.04		0.33				

(continued)

TABLE IX (continued)

R_F VALUES OF STEROIDS AND THEIR ACETATES

C_{21} Steroids	Chromatographic Solvent Systems						
	T.P.G.	B.F.	BL_1	D.	E_2B.	E_4	Bu.A
19-OH-DOC 19,21 diacetate	0.95		0.98				
18-OH-DOC			0.52				
18-OH-DOC 21 acetate			0.91				
Corticosterone	0.11		0.53	0.04	0.57		
Corticosterone 21 acetate	0.57		0.85	0.21	0.75		
Compound A	0.32	0.29		0.07	0.48	0.16	
Compound A 21 acetate	0.80	0.88		0.31	0.73	0.37	
DOC	0.59	0.80		0.44		0.48	
DOC 21 acetate	0.95	0.96	1.00	0.80		0.73	
C_{19} STEROID							
Testosterone	0.26	0.78		0.41		0.67	0.29
Testosterone acetate	0.98	1.00		1.00		0.92	0.92

TABLE IXa.

STEROID ACETATE TEST REACTIONS

Steroid	NaOHFl.	BT	H_3PO_4
18-OH-B 21 acetate	yellow	neg.	neg.
18-OH-B 18,21 diacetate	yellow	delayed	neg.
19-OH-B 19,21 diacetate	yellow	pos.	blue
Cortisol 21 acetate	yellow	pos.	yellow-green
Cortisone 21 acetate	yellow	pos.	neg.
19-OH-DOC 19,21 diacetate	blue-green	pos.	
18-OH-DOC 21 acetate	yellow	delayed	neg.
Corticosterone 21 acetate	yellow	pos.	yellow-green
Compound A 21 acetate	yellow	pos.	neg.
DOC 21 acetate	yellow	pos.	orange

Certain other steroids such as testosterone, progesterone and 11-keto-prog., also give this delayed faint color with the BT reagent. The reason for this remains obscure. All α-ketols give a rapid color formation with the BT reagent.

The Zimmermann reaction with various C_{19} steroids produced a variety of colors of different intensities. Testosterone not being a 17 ketosteroid yielded a dark purple color in this reaction, whereas epitestosterone did not react at all. The hydroxyl group at C-19 inhibits the Zimmermann reaction and 19-OH-testosterone does not give any color. The color obtained with 19-aldo-androstenedione is markedly weaker than that with androstenedione. A pink purple color is obtained with 19-nortestosterone.

These observations indicate that the Zimmermann reaction can not be regarded as specific for the 17 keto group when applied to paper since testosterone and 19-nortestosterone also produce color.

3. Serial fractionation of conversion products from progesterone-4-^{14}C by rat adrenal glands:

Progesterone-4-^{14}C incubated with rat adrenal quarters for 3 hours was transformed to some 23 labelled derivatives. These compounds can be separated by serial fractionation in paper chromatographic systems.

An identical extract applied on a single Bush B_5

system yielded only 6 recognizable radioactive zones. Figure 3 shows the original radioautographs and the corresponding ultraviolet light photographs of the compounds separable by these two methods. For all experiments involving labelled precursors the serial paper chromatographic fractionation method has been used as outlined in Figure 4.

From the separated compounds only 9 have been characterized and these are:

zone ≠ 2 18-OH-B

zone ≠ 3 19-OH-B

zone ≠ 6 Aldosterone

zone ≠ 9 19-OH-DOC

zone ≠ 12 18-OH-DOC

zone ≠ 14 Corticosterone (B)

zone ≠ 16 11-dehydro-corticosterone (A)

zone ≠ 22 11-deoxy-corticosterone (DOC)

zone ≠ 24 Progesterone.

4. Characterization of ^{14}C labelled steroids formed by rat adrenal glands from progesterone-4-^{14}C:

a. 18-OH-B (zone ≠ 2, Figure 3) absorbed ultraviolet light at 240 mμ, and gave a yellow soda fluorescence reaction. The BT reduction was very slow and produced only a faint color.

Figure 3. Chromatographic patterns of steroids present in the incubation medium of rat adrenals using the Bush B_5 system and serial fractionation.

RAT ADRENAL INCUBATION MEDIA EXTRACT

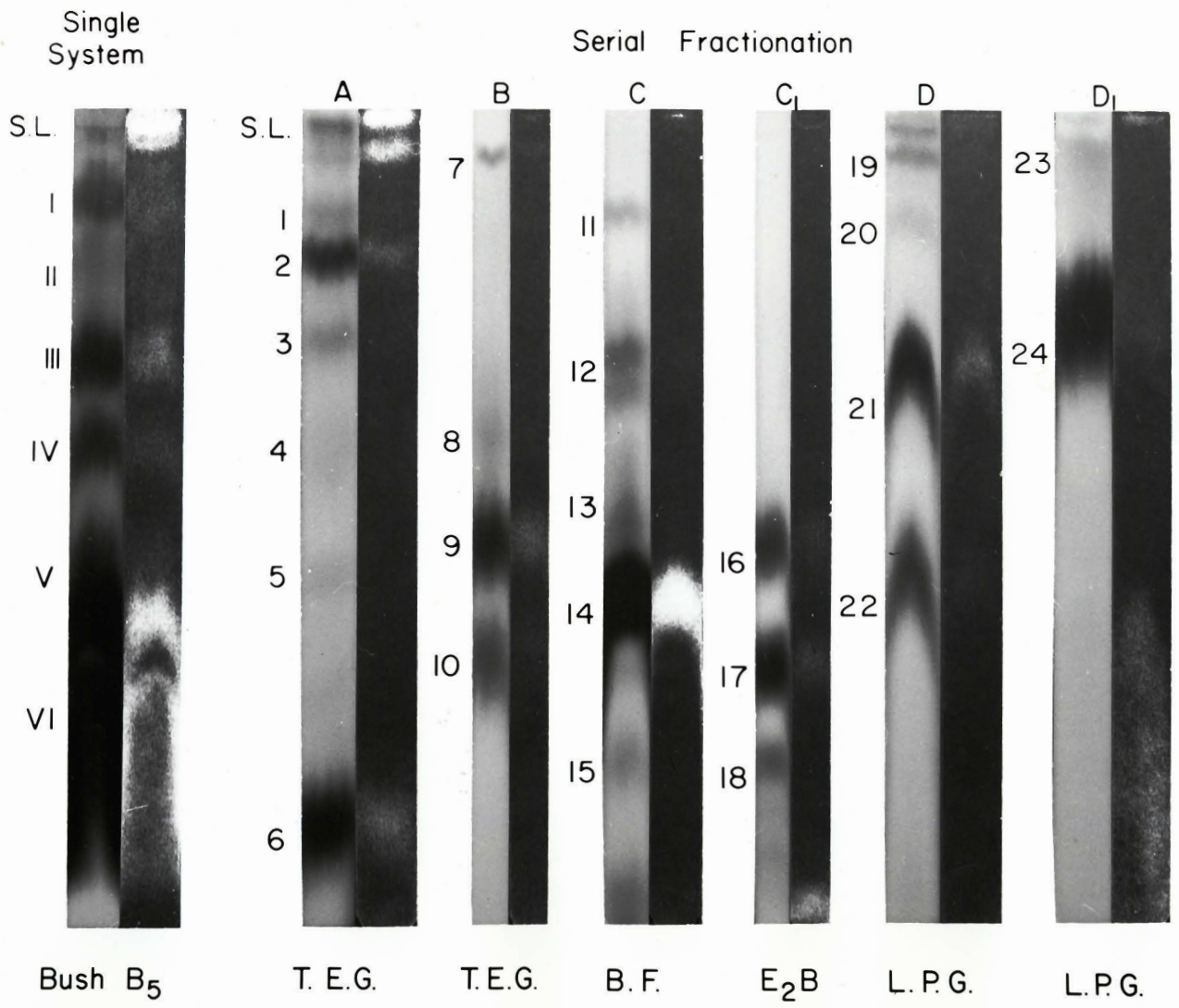
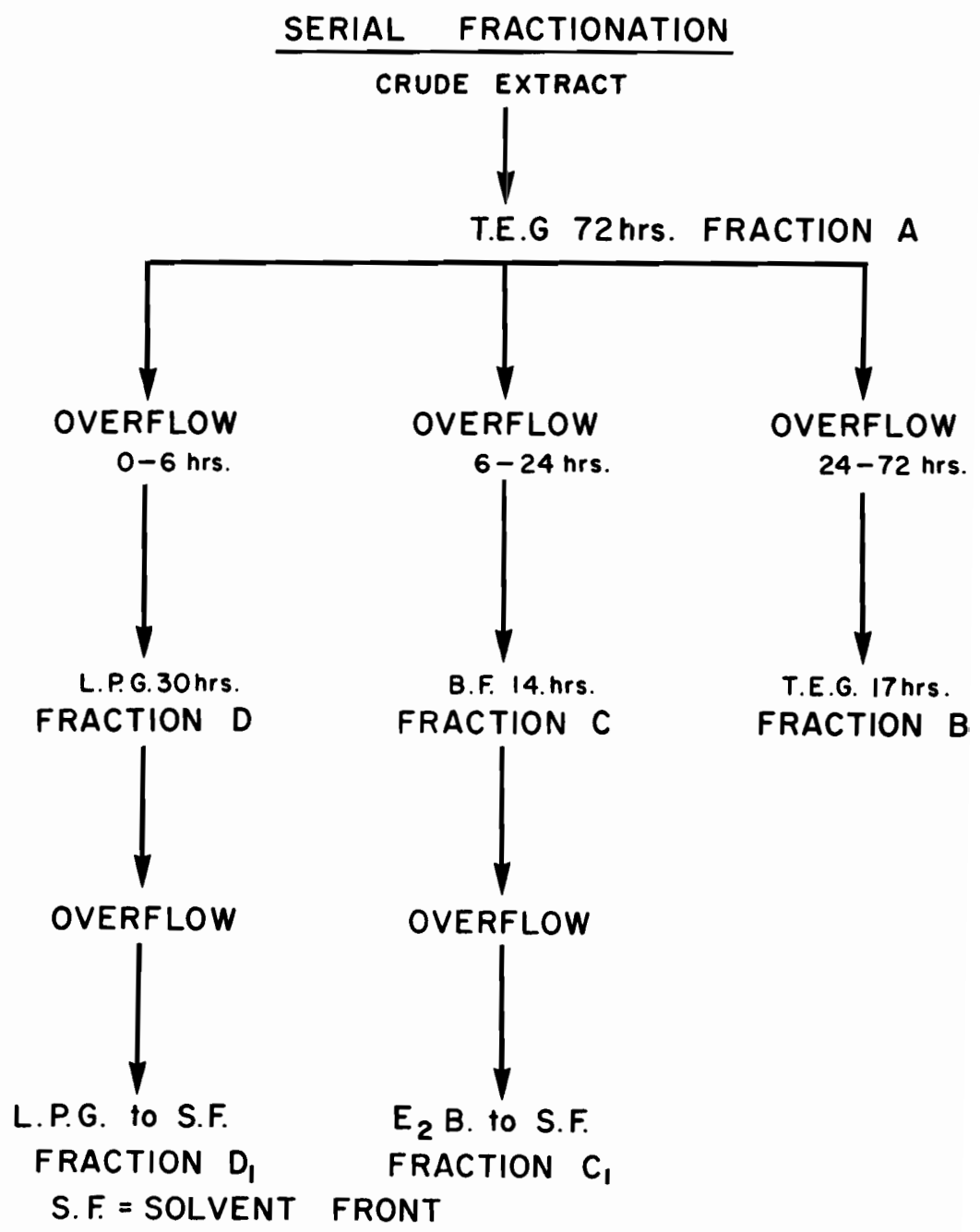


Figure 4. Scheme of serial fractionation.


SERIAL FRACTIONATION
CRUDE EXTRACT
T.E.G 72hrs. FRACTION A
OVERFLOW
0–6 hrs.
OVERFLOW
6–24 hrs.
OVERFLOW
24–72 hrs.
L.P.G.30hrs.
FRACTION D
B.F. 14.hrs.
FRACTION C
T.E.G. 17hrs.
FRACTION B
OVERFLOW
OVERFLOW
L.P.G. to S.F.
FRACTION D_1
E_2 B. to S.F.
FRACTION C_1
S.F. = SOLVENT FRONT

The eluted 18-OH-B material combined with authentic carrier was acetylated using 2 methods. Acetylation at room temperature for 18 hours yielded 30% of 18-OH-B 21-acetate and 70% of 18-OH-B 18,21-diacetate. The ratio of the mono and diacetates is changed if the reaction is carried out at 60°C for 15 minutes (Table X). The separation of the two 18-OH-B acetates was done in the B.L_1 system. As shown in Table X, the recovery of radioactivity after acetylation and chromatography ranged from 88.0 to 99.6%. Shortage of authentic reference standard prevented further studies.

b. 19-OH-B (zone # 3, Figure 3) has been consistently observed in all incubation experiments. This compound absorbed ultraviolet light at 240 mμ, reduced rapidly alkaline BT reagent and gave a yellow-greenish soda fluorescence. Pooled isolated 19-OH-B has been rechromatographed in T.E.G. system for 72 hours. From the pool, 2 major radioactive zones were separated (Figure 5): one which travelled as 19-OH-B, and a more polar zone which had a mobility similar to 18-OH-B. The latter zone on successive rechromatography in B.F. and $CHCl_3$F. systems behaved as a single compound and had the same mobility as 18-OH-B. On acetylation at 60°C for 15 minutes, this material yielded a single acetate which had the same chromatographic behaviour of 18-OH-B 21-acetate in the B.L_1 and T.P.G. systems.

Figure 5. Characterization of 19-OH-B.

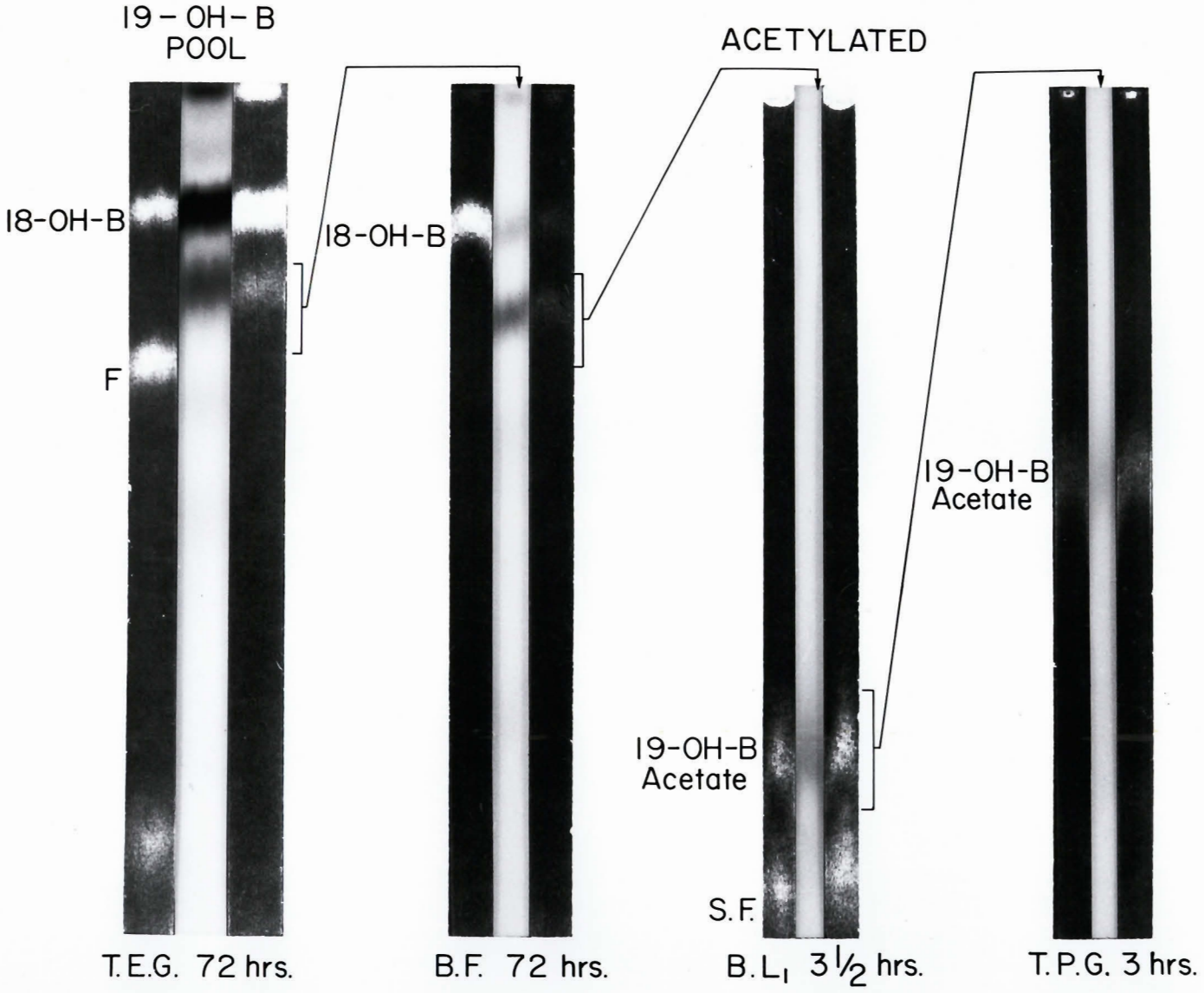

19 – OH – B
POOL
18-OH-B
F
T.E.G. 72 hrs.
18-OH-B
B.F. 72 hrs.
ACETYLATED
19-OH-B
Acetate
S.F.
B.L1 3½ hrs.
19-OH-B
Acetate
T.P.G. 3 hrs.

TABLE X

ACETYLATION OF 18-OH-B

Free compound cpm	Acetylation at 25°C		Acetylation at 60°C		Recovery %
	monoacet.	diacet.	monoacet.	diacet.	
75,400	23%	77%	-	-	97.7
66,300	29%	71%	-	-	99.6
315,450	-	-	71%	29%	98.0
57,390	-	-	67%	33%	91.0
44,830	-	-	69%	31%	97.0
7,660	-	-	71%	29%	88.0

The identity of this compound remains obscure. The 19-OH-B zone from T.E.G. system (Figure 5) was further purified in the B.F. system where 3 radioactive zones appeared, one having the mobility of 18-OH-B, another which behaved as 19-OH-B and a third less polar zone. The purified 19-OH-B zone on further chromatography in $CHCl_3F$. and E_2B. behaved as a single compound and had the mobility of authentic 19-OH-B. For further identification the radioactive 19-OH-B was combined with 50 µg of reference standard and acetylated at 60°C for 15 minutes. The acetylated 19-OH-B yielded only one zone in the $B.L_1$ and T.P.G. systems. The radioactivity did not separate from the ultraviolet light absorbing carrier.

c. Aldosterone (zone ≠ 6, Figure 3) absorbed ultraviolet light at 239 mµ, reduced rapidly alkaline BT solution, gave a yellow soda fluorescence and in the Mattox Porter-Silber reaction formed a chromogen with two maxima at 285 mµ and 400 mµ. The radioactivity remained associated with authentic aldosterone in Bush C and Bush B_5 systems.

d. 19-OH-DOC (zone ≠ 9, Figure 3) absorbed ultraviolet light at 240 mµ, reduced rapidly alkaline BT solution and gave a characteristic blue-green soda fluorescence. Fraction B was pooled from several experiments and on rechromatography yielded in the T.E.G. system 3 radioactive

zones (Figure 6). The zone which had the same mobility as authentic 19-OH-DOC was eluted and combined with 40 µg of authentic carrier and acetylated at 60°C for 15 minutes. The acetylated product remained homogeneous on chromatography in the B.L_1 and T.P.G. systems. After elution and hydrolysis with sodium carbonate the 19-OH-DOC acetate was transformed to free 19-OH-DOC and the radioactivity after chromatography in the T.P.G. system remained with the carrier (Figure 6).

e. 18-OH-DOC (zone ≠ 12, Figure 3) absorbed ultraviolet light and gave a yellow soda fluorescence. The BT reduction was very slow and yielded a faint color. On acetylation at 60°C the ^{14}C labelled 18-OH-DOC yielded one acetate, which remained associated with authentic 18-OH-DOC 21-acetate in the B.L_1 chromatographic system.

f. Corticosterone (zone ≠ 14) was ultraviolet light absorbing, it reduced rapidly alkaline BT reagent and gave a yellow soda fluorescence. In the Mattox Porter-Silber reaction this compound formed a chromogen with light absorption maxima at 285 mµ and 420 mµ similar to authentic corticosterone. The spectrum in sulfuric acid of the isolated corticosterone was identical with that of authentic corticosterone (Figure 7). Isolated ^{14}C labelled corticosterone on successive chromatography in B.F., E_2B., and Bush C systems the ^{14}C labelled corticosterone remained homogeneous and behaved as authentic

Figure 6. Characterization of 19-OH-DOC.

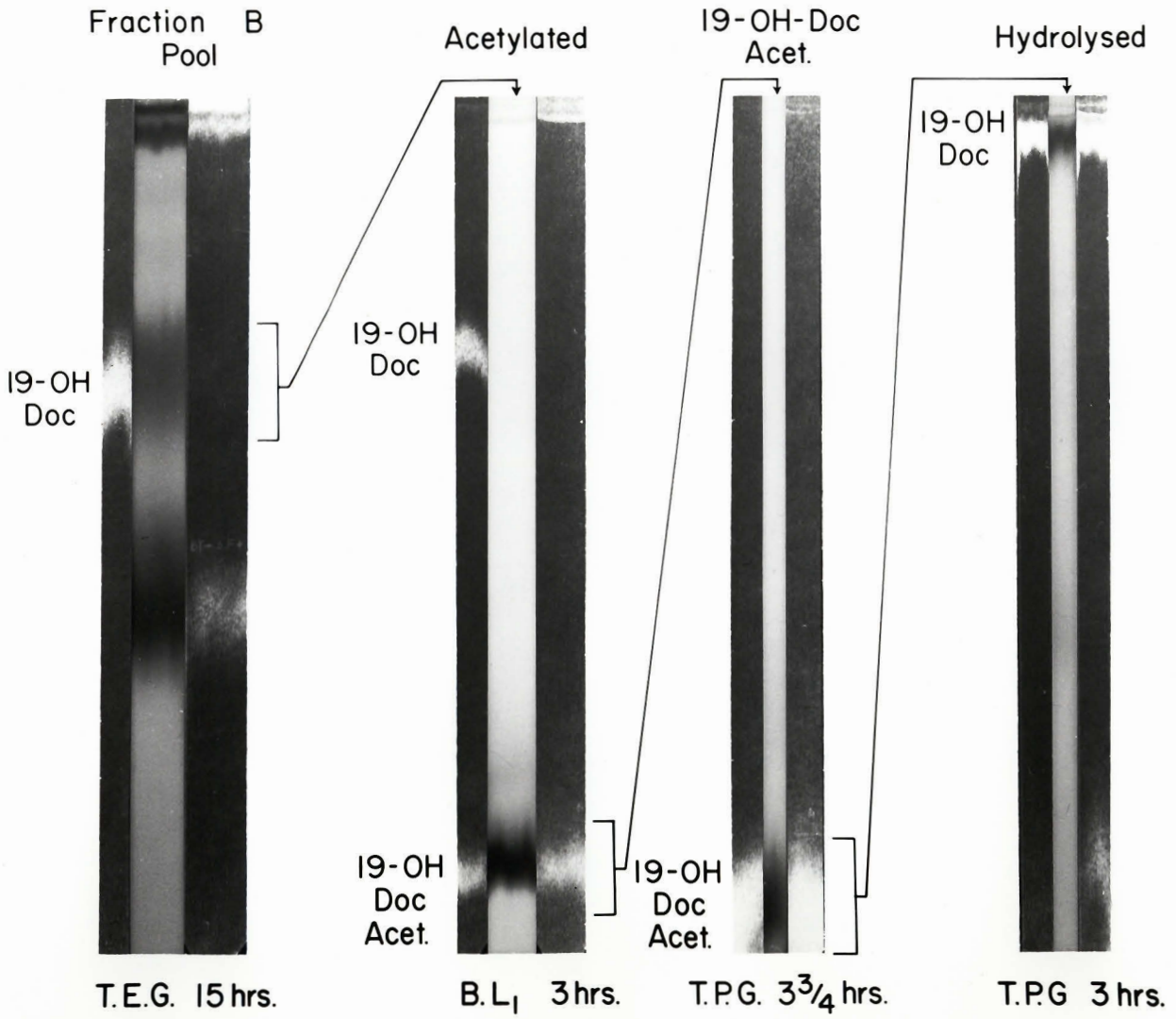
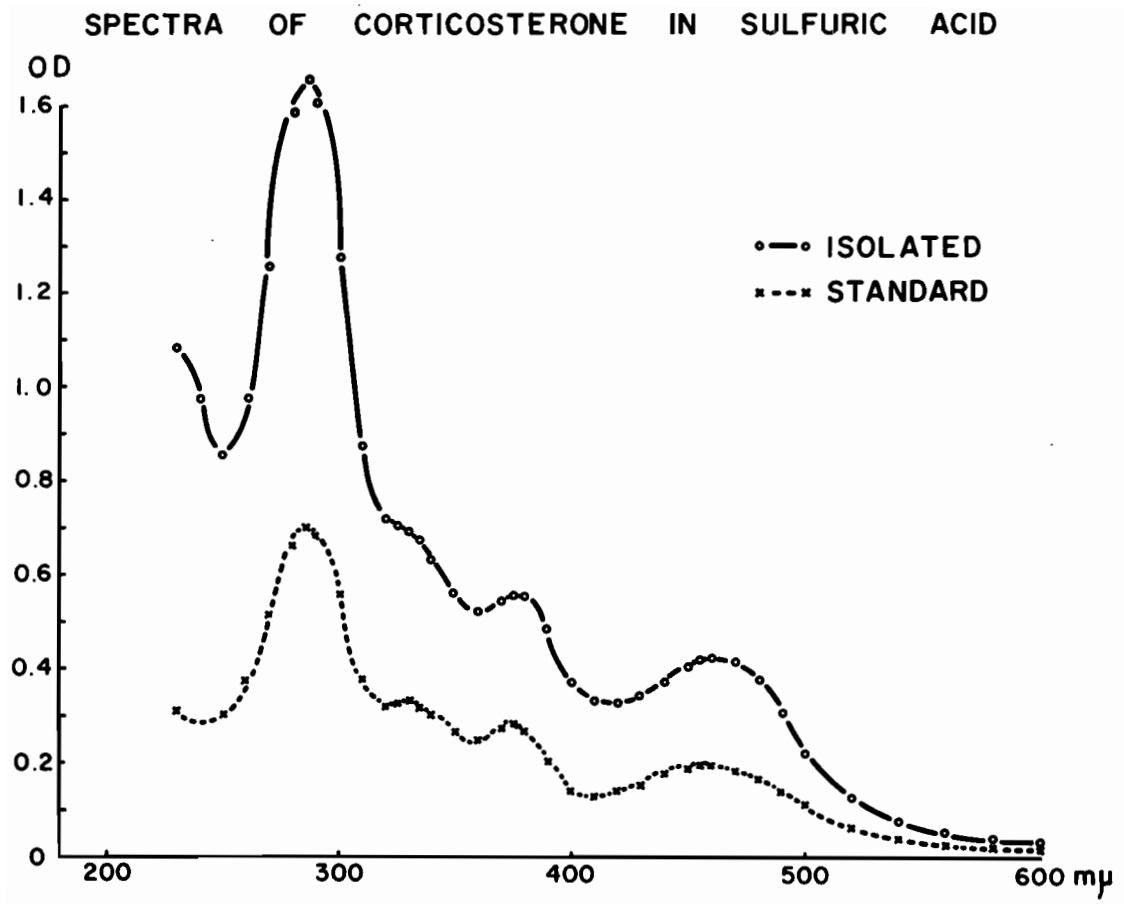

Fraction B
Pool
19-OH
Doc
T.E.G. 15 hrs.
Acetylated
19-OH
Doc
19-OH
Doc
Acet.
B.L1 3 hrs.
19-OH-Doc
Acet.
19-OH
Doc
Acet.
T.P.G. 3¾ hrs.
Hydrolysed
19-OH
Doc
T.P.G 3 hrs.

Figure 7. Spectra of corticosterone in sulfuric acid.


SPECTRA OF CORTICOSTERONE IN SULFURIC ACID
OD
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
200
300
400
500
600 mμ
ISOLATED
STANDARD

corticosterone. On acetylation at 60°C the corticosterone 21-acetate yielded on chromatography in the T.P.G. system a single radioactive zone with the same mobility as authentic corticosterone 21-acetate. This zone on hydrolysis with sodium carbonate solution yielded free corticosterone which had the same chromatographic mobility in the E_2B. system as the standard.

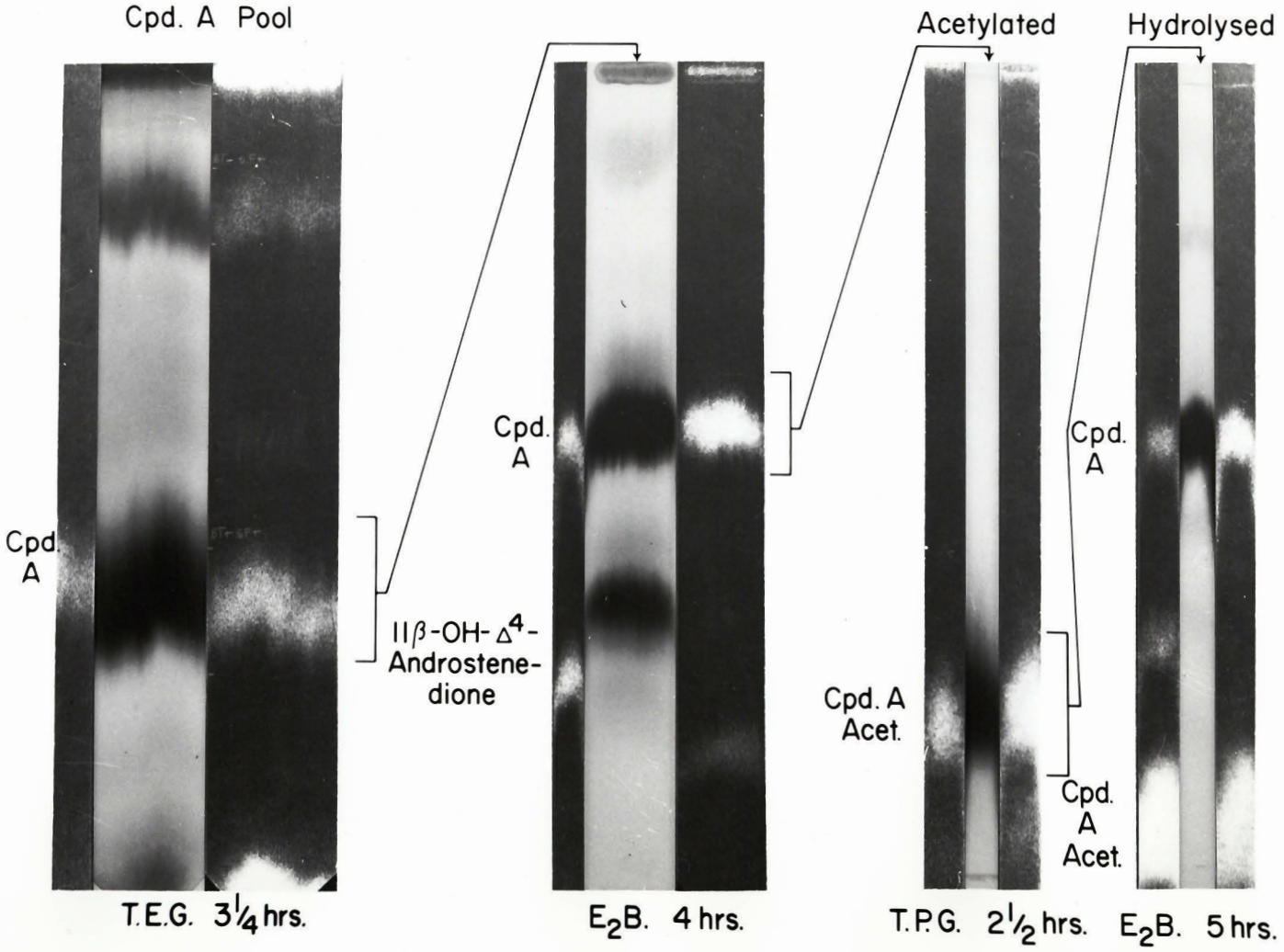
g. 11-dehydro-corticosterone (zone ≠ 16) absorbed ultraviolet light, reduced alkaline BT solution and gave yellow soda fluorescence.

Material from several experiments and different chromatographic systems was pooled and on rechromatography in the T.E.G. system yielded several radioactive zones (Figure 8). After purification in the E_2B. system and acetylation at 60°C the compound A 21 acetate was obtained which yielded a single substance in the T.P.G. system, having the same mobility as the authentic acetate. After elution and hydrolysis the free compound A was obtained and it gave a single zone which had a mobility similar to the standard compound A in the E_2B. system.

The zone ≠ 16 obtained after serial fractionation (Figure 3) was a highly purified fraction of compound A which showed no other compounds on rechromatography.

h. 11-deoxy-corticosterone (zone ≠ 22, Figure 3) absorbed ultraviolet light, reduced alkaline BT solution

Figure. 8. Characterization of compound A.


Cpd. A Pool
Cpd. A
T.E.G. 3¼ hrs.
Cpd. A
11β-OH-Δ4-Androstene-dione
E2B. 4 hrs.
Acetylated
Cpd. A Acet.
T.P.G. 2½ hrs.
Hydrolysed
Cpd. A
Cpd. A Acet.
E2B. 5 hrs.

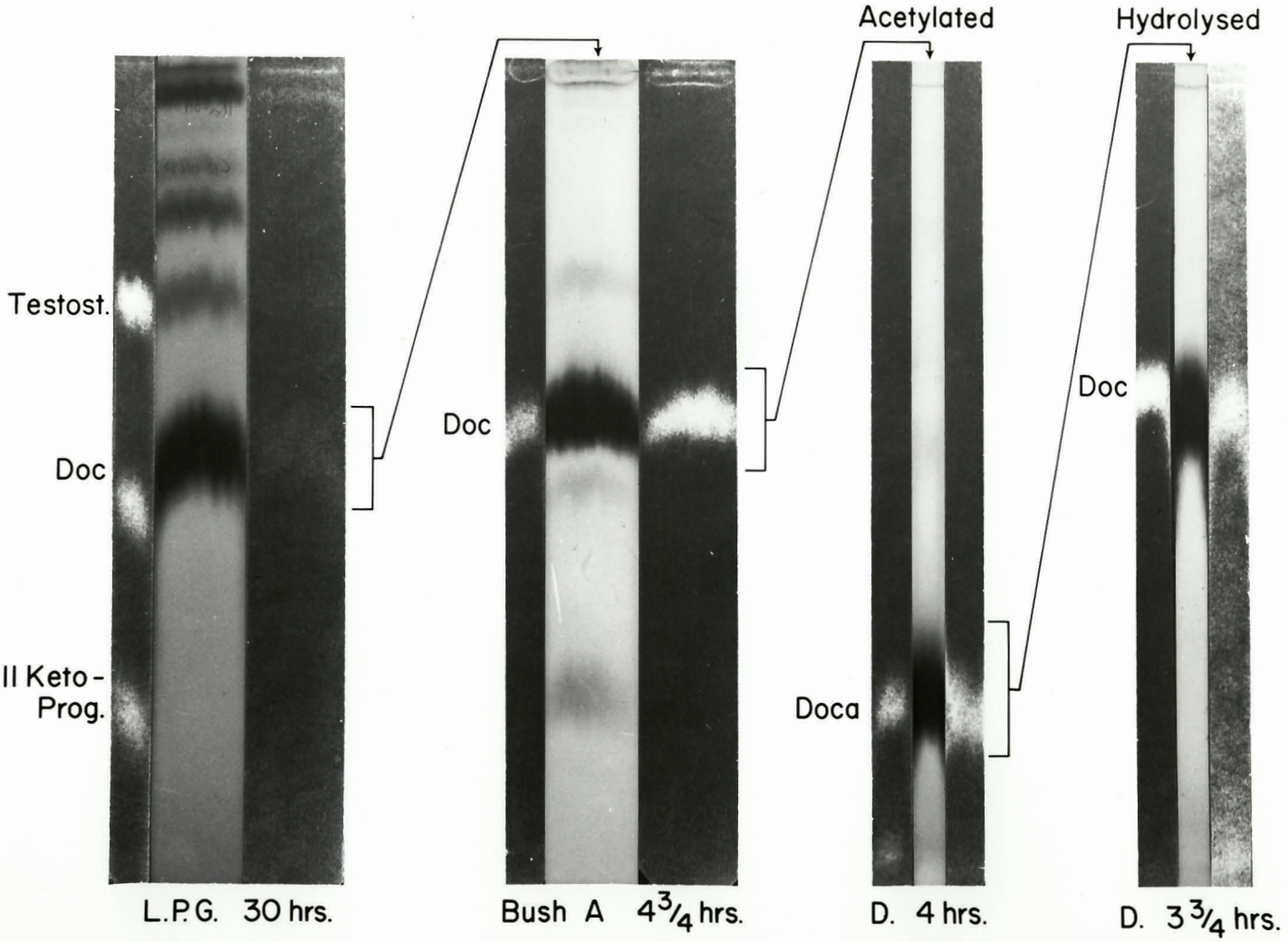
rapidly and gave a yellow soda fluorescence reaction. The pooled isolated DOC obtained from several experiments after chromatography in various systems was rechromatographed in the L.P.G. system for 30 hours. A major band showed properties of authentic DOC. Several minor more polar components were present (Figure 9). The material, with the chromatographic mobility of DOC was combined with 40 µg of authentic carrier and chromatographed in the Bush A system. It yielded pure DOC, which on acetylation at 60°C formed DOC 21-acetate. In system D this acetate remained together with the carrier. On hydrolysis of DOC 21-acetate, free DOC was obtained and it gave a single zone on rechromatography in system D.

Progesterone (zone # 24, Figure 3) is the unconverted precursor used in the incubation studies.

i. Unidentified compounds. Zone # 1, Figure 3, is an ultraviolet light absorbing material, giving a weak delayed reduction of alkaline BT reagent. This material had a chromatographic mobility similar to 16α-OH-B. The sulfuric acid spectrum was different from that of 16α-OH-B. Further studies have not been done with this compound.

Zone # 4 had the same chromatographic mobility as cortisol. A pool of this material was combined with

Figure 9. Characterization of DOC.


Acetylated
Hydrolysed
Testost.
Doc
II Keto-
Prog.
Doc
Doca
Doc
L.P.G. 30 hrs.
Bush A 4 3/4 hrs.
D. 4 hrs.
D. 3 3/4 hrs.

30 µg of cortisol as carrier and subsequently oxidized with sodium bismuthate. The cortisol was converted to 11β-OH-androstenedione whereas the radioactive material yielded 2 zones on chromatography in system D. One zone was more polar and the other slightly less polar than the oxidation product of the carrier. This result indicates that the material from zone # 4 was not identical with cortisol, however the radioactive material apparently underwent oxidation. The material in zone # 4 did not absorb ultraviolet light and did not reduce BT reagent nor did it produce soda fluorescence. It is possible that the quantity was too small for these test reactions.

Zone # 5 absorbed ultraviolet light, and showed a delayed reduction of alkaline BT reagent. It did not exhibit any soda fluorescence.

Zone # 7 was also ultraviolet positive and gave a delayed reduction of alkaline BT reagent. Soda fluorescence could not be demonstrated.

Zone # 8 appeared only occasionally and was not investigated.

Zone # 10 which was less polar than 19-OH-DOC was consistently found in the incubation media. Chromatographically it behaved as estradiol-17β . This zone absorbed ultraviolet light, reduced rapidly alkaline BT reagent

and produced a yellow soda fluorescence. It is doubtful that this material is an estrogen because of the positive soda fluorescence reaction, suggesting the presence of a $\Delta 4$-3 keto structure. Identity of this material remains unknown.

Zone # 11 appeared only occasionally and was not investigated.

Zone # 13 did not absorb ultraviolet light; it reduced alkaline BT reagent rapidly and did not give soda fluorescence. It is possible that this material is a reduced product of the pregnane series. Estrogen ketols which also reduce alkaline BT reagent are more polar in this system than the compound from zone # 13.

Zone # 15 was not investigated.

Zone # 17 showed ultraviolet light absorbing properties and in the E_2B. system it ran parallel to adrenosterone and dihydro-A. However, in the original T.E.G. system adrenosterone appeared in the overflow from 0 - 6 hours together with DOC. and would not be expected to be present in fraction C_1 (Figure 3). This compound was not dihydro-A because of the ultraviolet light absorption, positive soda fluorescence and negative BT reaction.

Zone # 18 had a mobility similar to 11β-OH-androstenedione and 16α-OH-prog. It did not absorb ultraviolet

light nor did it give soda fluorescence, but it reduced rapidly alkaline BT reagent. The homogeneity of zone ≠ 18 has been investigated.

This material was combined with 40 µg of 11β-OH-androstenedione as a carrier and the pool was subjected to chromium trioxide oxidation. On chromatography in system D. only 5% of the radioactivity remained associated with adrenosterone. The remainder of the radioactivity appeared as a single zone more polar than adrenosterone having the same mobility as 11β-OH-androstenedione but it reduced alkaline BT reagent rapidly and did not give any soda fluorescence reaction. The phosphoric acid reaction produced a faint blue color suggesting a keto group at C-11 (12δ). The amount of radioactivity associated with adrenosterone was too low for further investigations. These observations suggested that the material in zone ≠ 18 was oxidizable and may contain a small amount of 11β-OH-androstenedione. The negative soda fluorescence reaction of the other substance suggested the absence of a Δ^4-3 keto structure and the reduction of BT reagent the possibility of an α-ketol structure.

Zone ≠ 19 failed to absorb ultraviolet light and did not react with BT reagent. It had a mobility similar to 6β-OH-prog. On chromatography with 30 µg of authentic

carrier in L.P.G. system for 72 hours the radioactivity separated from the carrier.

Zone # 20 did not react with BT reagent and failed to absorb ultraviolet light. It behaved chromatographically as 11β-OH-prog. On chromatography in L.P.G. system for 72 hours the radioactivity separated from the authentic carrier by 1 cm.

Zone # 21 absorbed ultraviolet light, gave a faint delayed color with BT reagent and produced a yellow soda fluorescence reaction. It had the chromatographic mobility similar to 17α-OH-prog., testosterone and 19-nortestosterone. A pool of the material in zone # 21 was rechromatographed in L.P.G. system for 30 hours. After elution it was combined with 89.5 mg of authentic 17α-OH-prog., and crystallized in benzene:methanol solution (Table XI). After the first crystallization, most of the radioactivity remained in the mother liquor. The crystals on repeated crystallization constantly lost radioactivity, indicating that the material from zone # 21 was not identical with 17α-OH-prog.

A portion of the material in zone # 21 was rechromatographed in the T.P.G. system for 3 hours and the radioactive zone showed the same mobility as testosterone. This material after elution was acetylated and rechromatographed in Bush A system. It yielded only one compound

TABLE XI

CONVERSION PRODUCT IN THE 17α-OH-PROG.-TESTOSTERONE AREA: ITS NON-IDENTITY TO AUTHENTIC 17α-OH-PROG. AFTER CRYSTALLIZATION

Solvent: Benzene:methanol (3:1)		
Authentic 17α-OH-prog. 89.5mg		
Isolated ^{14}C compound (zone # 21)	46,420	cpm
Initial specific activity	519	cpm/mg
Mother liquor	404	cpm/mg
First crystals	75	cpm/mg
Second crystals	24	cpm/mg
Third crystals	14	cpm/mg

which had the same mobility as testosterone acetate. Testosterone acetate was combined with the acetylated sample and applied in the E_4 system. The radioactivity separated from the carrier by 0.5 cm. This acetate was further chromatographed on a two-dimensional TLC system. As shown in Figure 1, the radioactivity definitely separated from the testosterone acetate carrier, thus disproving theidentity with this steroid.

Another portion of this material (zone # 21) was combined with 30 µg of 19-nortestosterone and chromatographed in the T.P.G. system. The radioactive zone was less polar than the carrier and clearly separated from it.

The material in zone # 23 failed to react with the BT reagent and was not visible under ultraviolet light. This zone was eluted and combined with 30 µg of androstenedione. On rechromatography in Bush A system, the radioactivity separated from the carrier.

5. Metabolism of progesterone-4-^{14}C by rat adrenal glands:

a. Distribution of radioactivity in vitro: Rat adrenal quarters have been incubated with progesterone-4-^{14}C for 15 minutes, 60 minutes and 180 minutes. The radioactive material extracted from the incubation medium and the tissue has been assayed and expressed as a percentage of added radioactivity. Table XII and Table XIII. The total recovery of radioactivity ranged from 91 to 98%.

TABLE XII

CONVERSION OF PROGESTERONE-4-^{14}C BY RAT ADRENAL GLANDS

	Exp. 1		Exp. 2	
Adr. tissue	0.372 gm		0.385 gm	
Prog.-4-^{14}C	1.90x10^6 cpm/20.2 µg		1.90x10^6 cpm/20.2 µg	
Inc. time	15 minutes		60 minutes	
	Inc.medium	Tissue	Inc. medium	Tissue
Extracted cpm:	1.43x10^6	0.42x10^6	1.38x10^6	0.39x10^6
Recovered:	75.3%	22.1%	72.5%	20.3%
COMPOSITION:	%	%	%	%
Zone ≠ 1	0.5	-	0.9	-
18-OH-B	0.9	0.5	2.3	1.4
19-OH-B	0.5	-	1.2	-
Aldosterone	0.9	0.8	2.4	1.5
19-OH-DOC	0.3	0.5	0.6	0.7
Zone ≠ 10	0.2	0.4	0.3	-
18-OH-DOC	4.5	5.4	6.5	3.2
B	15.9	15.5	22.9	15.2
A	0.8	2.7	1.2	3.8
DOC	8.0	11.6	9.7	11.1
Zone ≠ 23	0.8	2.8	0.8	2.2
Prog.	46.7	31.4	27.4	25.0

TABLE XIII

CONVERSION OF PROGESTERONE-4-^{14}C BY RAT ADRENAL GLANDS

	Exp. 1		Exp. 2	
Adr. tissue	0.610 gm		0.550 gm.	
Prog.-4-^{14}C	1.67x10^6 cpm/17.9 µg		2.42x10^6 cpm/152 µg	
Inc. time	180 minutes		180 minutes	
	Inc. medium	Tissue	Inc. medium	Tissue
Extracted cpm:	1.42x10^6	0.11x10^6	2.03x10^6	0.196x10^6
Recovered:	85.0%	6.6%	84.0%	8.1%
COMPOSITION:	%	%	%	%
Zone ≠ 1	1.5	-	1.3	-
18-OH-B	4.0	0.9	2.2	4.1
19-OH-B	0.6	-	1.1	-
Zone ≠ 4	0.7	-	0.8	-
Zone ≠ 5	-	-	0.7	-
Aldosterone	7.1	1.4	6.1	6.7
19-OH-DOC	1.1	0.8	3.8	-
Zone ≠ 10	4.8	0.9	1.8	-
18-OH-DOC	2.3	1.5	1.9	7.2
Zone ≠ 13	0.7	-	1.7	-
B	24.6	10.9	28.8	22.7
Zone ≠ 15	-	-	1.9	-
A	1.9	-	2.0	0.2
Zone ≠ 17	-	-	3.8	-
Zone ≠ 18	-	-	0.1	0.1
Zone ≠ 19	-	-	0.8	-
Zone ≠ 20	-	-	0.5	-
Zone ≠ 21	-	-	5.4	-
DOC	10.5	16.9	3.4	3.7
Zone ≠ 23	-	-	0.7	-
Prog.	18.5	21.0	16.0	20.1

The tissue after an incubation period of 15 minutes contained 22.1% of added radioactivity. On longer incubation the radioactivity extractable from the tissue decreases. After an incubation period of 60 minutes the tissue contained 20.3% and after 180 minutes 6.6% and 8.1% of the added radioactivity. The retention of ^{14}C by the rat adrenal tissue at various times of incubation is shown in Figure 10. Adrenal quarters in vitro rapidly take up the labelled steroid and on longer incubation release the labelled products. Adrenal glands from hypophysectomized rats retained more radioactivity than glands from normal rats after an incubation period of 180 minutes.

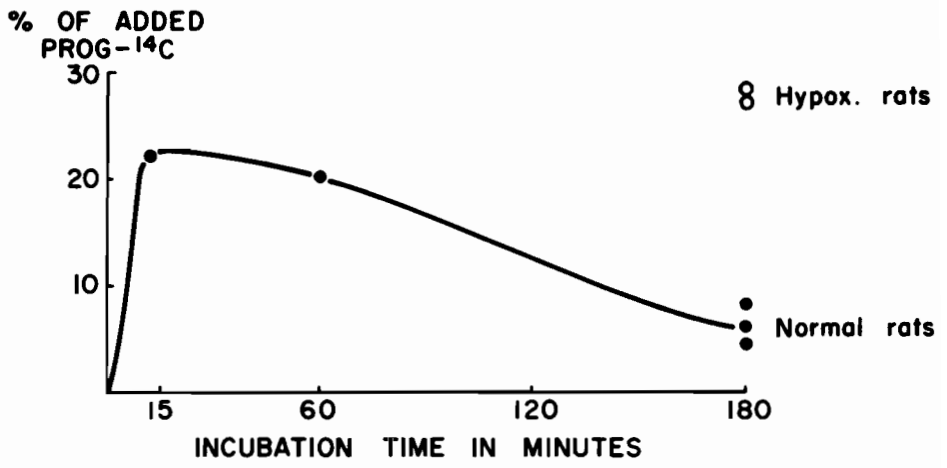
b. Changes in progesterone concentration during incubation: The extracted radioactive material has been chromatographed using the described serial fractionation method. The isolated progesterone is expressed as a percentage of the total extractable radioactivity from the incubation media and from the tissue after incubation for 15, 60 and 180 minutes. The results are shown in Figure 11. At 15 minutes the incubation medium contained 46.7% unconverted progesterone, and the tissue 31.4% unconverted progesterone. On longer incubation the percentage of unconverted progesterone declined in the incubation medium and also in the tissue.

The total conversion of the precursor, progesterone-

Figure 10. Retention of ^{14}C by rat adrenal tissue after incubation with progesterone-4-^{14}C.

Figure 11. Relative concentration of progesterone-4-^{14}C in the incubation media and in the tissue.

RETENTION OF ^{14}C BY RAT ADRENAL TISSUE



RELATIVE CONCENTRATION OF PROGESTERONE-4-^{14}C

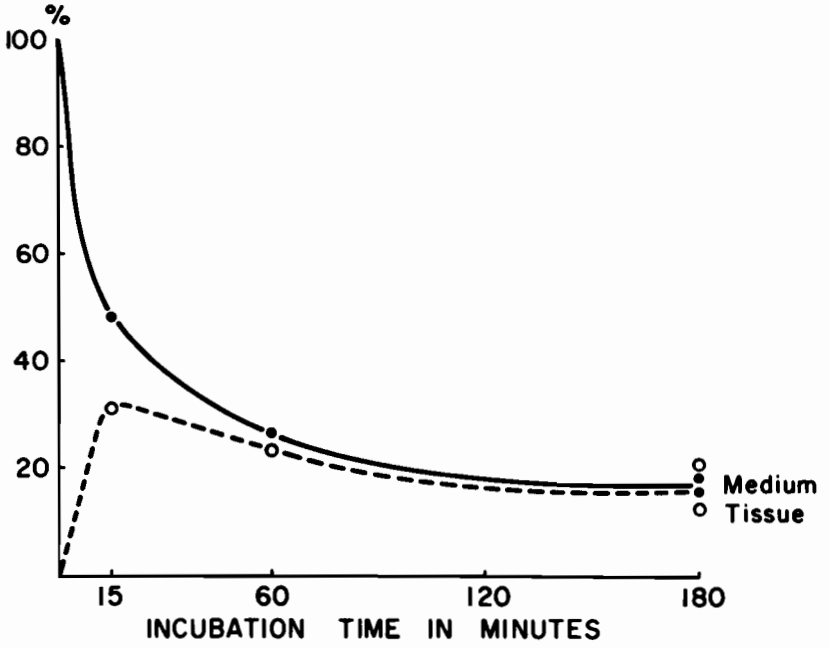
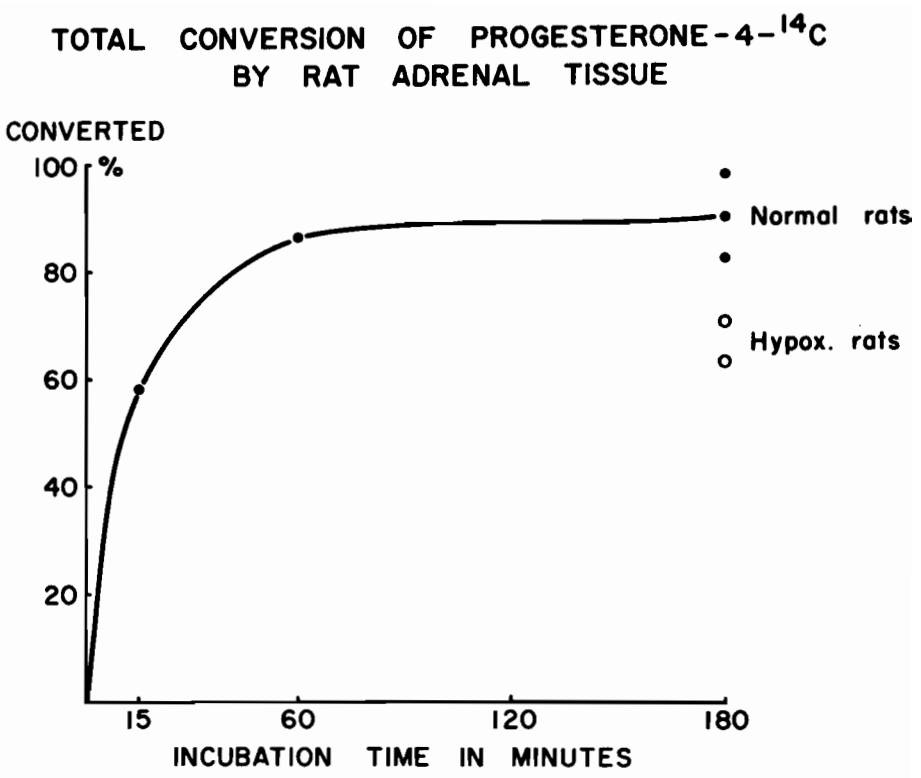


Figure 12. Total conversion of progesterone-4-^{14}C by rat adrenal tissue after different periods of incubation.

Figure 13. Composition of incubation media after various periods of incubation.


TOTAL CONVERSION OF PROGESTERONE-4-14C
BY RAT ADRENAL TISSUE
CONVERTED
100 %
80
60
40
20
Normal rats
Hypox. rats
15
60
120
180
INCUBATION TIME IN MINUTES

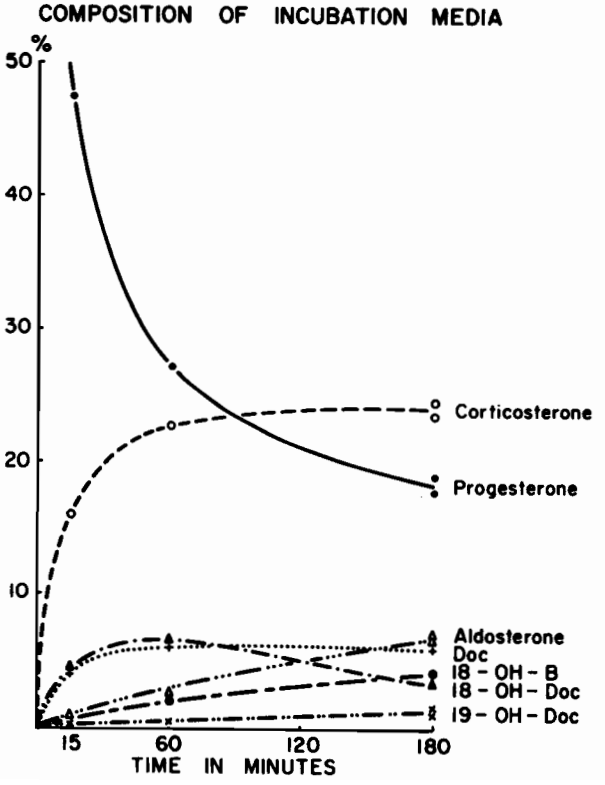

COMPOSITION OF INCUBATION MEDIA
%
50
40
30
20
10
Corticosterone
Progesterone
Aldosterone
Doc
18 - OH - B
18 - OH - Doc
19 - OH - Doc
15
60
120
180
TIME IN MINUTES

TABLE XIV

EFFECT OF pH ON CONVERSION OF PROGESTERONE-4-^{14}C

	Exp. 1		Exp. 2	
Adr. tissue	2.253 gm		2.216 gm	
Prog.-4-^{14}C	7.05×10^6 cpm/706.0 µg		7.05×10^6 cpm/706.0µg	
Inc. time	180 minutes		180 minutes	
pH	7.4		8.5	
	Inc. medium	Tissue	Inc. medium	Tissue
Extracted cpm:	4.40×10^6	1.89×10^6	4.40×10^6	1.85×10^6
Recovered:	62.4%	26.8%	62.4%	26.2%
COMPOSITION:	%	%	%	%
Zone ≠ 1	1.4	1.1	1.5	1.2
19-OH-B	1.1	-	1.3	-
Zone ≠ 4	1.1	-	1.4	-
Aldosterone	7.3	2.0	7.5	1.0
Zone ≠ 8	0.8	-	0.8	-
19-OH-DOC	2.2	-	2.4	-
Zone ≠ 11	0.5	-	0.4	-
18-OH-DOC	lost	9.5	6.8	2.9
B	31.6	16.1	33.3	15.0
A	6.1	3.6	6.0	3.3
Zone ≠ 18	1.2	-	1.2	-
DOC	7.0	3.9	8.6	5.8
Prog.	2.8	4.6	3.9	8.4

≠ 19990a) has been incubated with adrenal quarters in the presence of progesterone-4-^{14}C for 180 minutes. Table XV and Table XVI. The conversion of progesterone precursor in the preparation containing angiotensin II was in the same order as that of the control preparation where angiotensin was omitted. The formation of conversion products in the presence of angiotensin II showed no significantly demonstrable change as compared to the control preparation.

f. Conversion of progesterone by adrenal glands from hypophysectomized rats: It has been shown by Venning and Lucis (26, 34) that growth hormone administration to hypophysectomized rats increased the endogenous formation of aldosterone by the adrenal glands in vitro. The effect of porcine growth hormone (P398A) prepared by Dr. Wilhelmi, has been studied in hypophysectomized rats.

On the tenth day after hypophysectomy a group of 13 animals were injected with 0.3 mg of growth hormone in normal saline for 3 days. The control group of 15 animals received saline injections only. Adrenalectomies were done 4 hours after the last injection under sodium pentobarbital anaesthesia. The arterial blood from both groups of animals was obtained from the abdominal aorta and analysed for serum sodium and potassium content. Table XVII. The mean serum sodium concentration in the control group was

TABLE XV

EFFECT OF ANGIOTENSIN II ON THE CONVERSION OF PROGESTER-ONE-4-^{14}C

	Exp. 1		Exp. 2	
Angiotensin II	None		400µg(4x100µg)	
Adr. tissue	0.610 gm		0.618 gm	
Prog.-4-^{14}C	1.67x10^{6}cpm/17.9µg		1.67x10^{6}cpm/17.9µg	
Inc. time	180 minutes		180 minutes	
	Inc. medium	Tissue	Inc. medium	Tissue
Extracted cpm:	1.42x10^6	0.11x10^6	1.40x10^6	0.08x10^6
Recovered:	85.0%	6.6%	84%	4.8%
COMPOSITION:	%	%	%	%
Zone ≠ 1	1.5	-	1.0	-
18-OH-B	4.0	0.9	4.9	3.0
19-OH-B	0.6	-	0.4	-
Zone ≠ 4	0.7	-	0.4	-
Aldosterone	7.1	1.4	7.1	-
19-OH-DOC	1.1	0.8	1.0	0.5
Zone ≠ 10	4.8	0.9	3.8	2.7
18-OH-DOC	2.3	1.5	2.9	1.2
Zone ≠ 13	0.7	-	1.0	-
B	24.6	10.9	23.0	16.0
A	1.9	-	2.2	-
DOC	10.5	16.9	7.4	10.9
Prog.	18.5	21.0	17.8	12.8

TABLE XVI

EFFECT OF ANGIOTENSIN II ON THE CONVERSION OF PROGESTERONE-4-^{14}C

	Exp. 1	Exp. 2
Angiotensin II	None	400 µg(4x100 µg)
Adr. tissue	0.518 gm	0.528 gm
Prog.-4-^{14}C	1.69x10^{6}cpm/18.0 µg	1.69x10^6 cpm/18.0 µg
Inc. time	180 minutes	180 minutes
	Inc. medium	Inc. medium
Extracted cpm:	1.22x10^6	1.66x10^6
Recovered:	72.2%	69.0%
COMPOSITION:	%	%
18-OH-B	6.2	5.7
Aldosterone	7.1	6.6
18-OH-DOC	3.7	2.7
B	23.9	26.6
DOC	9.5	2.3
Prog.	8.1	6.5

TABLE XVII

SERUM ELECTROLYTES IN HYPOPHYSECTOMIZED RATS

Control		Growth Hormone Treated	
Na mEq/l	K mEq/l	Na mEq/l	K mEq/l
151.0	4.3	149.0	4.6
152.5	4.4	146.0	3.8
142.5	4.4	144.0	4.6
142.5	4.8	146.0	4.7
150.0	4.2	142.5	4.5
137.0	4.5	149.0	5.0
146.0	3.9	144.0	3.7
129.0	4.4	142.0	3.3
152.5	5.4	--	-
Mean 144.7	4.48	145.3	4.25
S.E. ± 2.69	± 0.14	± 0.95	± 0.21

found to be 144.7 mEq/liter and that of the growth hormone treated animals 145.3 mEq/liter. Differences in the serum sodium level between both groups were not satistically significant. The serum potassium concentration in the control group had a mean value of 4.48 mEq/liter which is slightly higher than the mean potassium of 4.25 mEq/liter found in growth hormone treated animals. The difference between these two means, however, was not statistically significant.

Adrenal glands from control animals have an average weight of 6.43 mg/gland, whereas the adrenal glands from growth hormone treated rats were found to be heavier, 8.69 mg/gland. The average weight of the adrenal glands from normal rats (body weight 150 to 180 gm) was in the order of 12 to 13 mg/gland. Thus, 13 days after hypophysectomy the adrenal weight had decreased by almost one half. A slight increase in the adrenal weight after growth hormone treatment may be attributed to the effect of growth hormone. Similar but smaller weight increases of adrenal glands have been observed by Venning and Lucis (34).

Adrenal quarters from control and growth hormone treated rats have been incubated with 14.7 µg (1.42×10^6 cpm) progesterone-4-^{14}C for 180 minutes. After incubation, 98.8% of the added radioactivity was recovered. The composition of the extracted material from the incubation medium and the

tissue has been analyzed using serial fractionation technique. Table XVIII. Adrenal glands from growth hormone treated animals converted slightly more of the added progesterone than the control tissue.

The formation of 18-OH-B, aldosterone, 18-OH-DOC, B and DOC was slightly higher in the adrenals from growth hormone treated rats than in the adrenals from untreated animals, suggesting growth hormone exerted a stimulating effect in vivo on the adrenal gland by increasing its weight and by the activation of enzyme systems necessary for the biosynthesis of steroid hormones. The adrenal tissue from hypophysectomized rats metabolized progesterone in a different pattern than the adrenal tissue from normal rats. The sum of the mineralocorticoids, DOC and aldosterone, formed by the adrenals of the hypophysectomized rats was higher than the formation of corticosterone, whereas in the adrenal tissue from normal rats, the formation of corticosterone was higher than the sum of aldosterone and DOC. This difference is probably due to the rapid atrophy of the zona fasciculata and reticularis following hypophysectomy.

6. Conversion of DOC-4-^{14}C by rat adrenal glands:

The conversion of DOC-4-^{14}C by the normal rat adrenal has been studied. For comparison purposes, 2.492 gm of

TABLE XVIII

CONVERSION OF PROGESTERONE-4-^{14}C BY ADRENAL GLANDS FROM HYPOPHYSECTOMIZED RATS

	CONTROL		G.H. TREATED	
Number of rats	15		13	
Adr. tissue	0.180 gm		0.226 gm	
Prog.-4-^{14}C	1.42x10^{6}cmp/14.7µg		1.42x10^{6}cpm/14.7 µg	
Inc. time	180 minutes		180 minutes	
	Inc.medium	Tissue	Inc.medium	Tissue
Extracted cpm:	0.98x10^6	0.42x10^6	0.98x10^6	0.42x10^6
Recovered:	69%	29.8%	69.0%	29.8%
COMPOSITION:	%	%	%	%
Zone ≠ 1	1.5	--	1.6	-
18-OH-B	4.4	0.4	6.1	0.9
19-OH-B	0.5	--	0.7	--
Zone≠ 4	1.7	--	1.2	--
Aldosterone	4.9	--	5.6	--
19-OH-DOC	1.3	0.3	1.8	0.7
Zone ≠ 10	0.7	--	0.6	--
Zone ≠ 11	0.3	--	0.5	--
18-OH-DOC	1.7	--	3.6	--
B	8.8	1.0	12.8	3.3
A	3.0	--	3.8	--
DOC	7.1	2.7	10.1	3.2
Prog.	32.0	42.8	28.0	29.7

adrenal quarters were incubated with 30 µg of DOC and 0.68 gm of tissue with 210 µg DOC. Table XIX. Both preparations converted the DOC precursor to a similar extent. The conversion products derived from DOC were similar in number and in their chromatographic mobilities as those formed from progesterone. In Table XIX only 12 major radioactive zones have been assayed. From DOC the principle conversion product was corticosterone. Increasing the amount of DOC precursor (210 µg) resulted in increased conversion to corticosterone and a decreased formation of 18-OH-B and aldosterone. As compared with progesterone, 30 µg DOC yielded more radioactive 18-OH-B and aldosterone, whereas the formation of 18-OH-DOC was not altered. These observations show that the weight of DOC precursor alters the pattern of biosynthesis in vitro and suggest that DOC can function as an intermediate steroid in the conversion of progesterone to other steroids. The final identified and unidentified products have chromatographically a higher polarity than DOC with the exception of material in zone # 23, which is less polar than DOC (Figure 3). No radioactivity was found in the area of progesterone.

7. Effect of angiotensin II on endogenous formation of steroids by the rat adrenal:

Angiotensin II (Ciba preparation 19990a) has been

TABLE XIX

CONVERSION OF DOC-4-^{14}C BY RAT ADRENAL GLANDS

	Exp. 1		Exp. 2	
Adr. tissue	2.492 gm		0.680 gm	
DOC-4-^{14}C	4.20x10^{6}cpm/30 µg		1.40x10^6 cpm/210 µg	
Inc. time	180 minutes		180 minutes	
	Inc. medium	Tissue	Inc. medium	Tissue
Extracted cpm:	2.98x10^6	0.76x10^6	1.03x10^6	0.28x10^6
Recovered:	70.9%	18.0%	73.6%	20.2%
COMPOSITION:	%	%	%	%
Zone ≠ 1	2.0	2.6	2.3	2.7
18-OH-B	10.6	5.9	5.5	2.7
19-OH-B	3.7	-	3.1	-
Aldosterone	11.8	5.6	7.6	2.8
19-OH-DOC	1.5	1.7	1.1	1.9
Zone ≠ 10	3.9	5.9	3.6	3.4
Zone ≠ 11	0.4	-	0.3	-
18-OH-DOC	2.1	1.2	2.4	2.4
Zone ≠ 13	2.1	0.9	1.9	1.1
B	26.0	28.4	32.0	28.7
A	3.7	4.9	4.6	2.1
DOC	6.2	9.4	12.6	22.6
Radioactivity accounted for	74.0%	66.5%	77.0%	70.4%

assayed for its stimulating activity on corticosteroidogenesis.

Rat adrenal quarters were pre-incubated for 30 minutes in 15 ml KRG using siliconized glass vessels. The solution was changed and the incubation continued for an additional 2 hours with and without addition of angiotensin II. In most instances, angiotensin II was added to the test samples in divided doses every 30 minutes. At the end of the incubation period the steroid hormones released into the incubation media were extracted and chromatographically separated. Individual steroid hormones were assayed using the INH and Mattox Porter-Silber reactions. The amount of steroid hormone produced has been expressed as a rate of secretion in micrograms per gram of tissue per one hour of incubation time. Table XX. Angiotensin at a dose level of 100 µg failed to alter the endogenous steroid formation. Slight increases in formation of corticosterone were observed with 200 to 500 µg of angiotensin II. Increases in formation of aldosterone and 18-OH-DOC have been obtained with 400 and 500 µg of angiotensin II. The most pronounced increase at these dose levels was obtained with 18-OH-DOC.

The effect of angiotensin on rat adrenal glands has also been studied in the presence of NADP and glucose-6-phosphate. Table XXI. The addition of 4.6 µM of NADP and 13.8 µM of glucose-6-phosphate resulted in a marked increase in the secretion of B and in a smaller increase in the secretion of aldosterone and 18-OH-DOC. The formation of 18-OH-B remained unaltered. The addition of angiotensin II

TABLE XX

EFFECT OF ANGIOTENSIN II ON RAT ADRENAL GLANDS IN VITRO

Exp. #	Angiotensin II	Adrenal Tissue gm	Steroid Hormones µg/gm/hr			
			18-OH-B	ALDO	18-OH-DOC	B
1	None	0.250	1.0	10.4	3.8	16.8
	100µg(single dose)	0.278	0.9	9.6	3.4	16.0
2	None	0.556	-	12.5	-	21.5
	200µg(2x100 µg)	0.540	-	11.9	-	25.0
3	None	0.496	8.1	9.1	-	31.8
	200µg(4x50 µg)	0.480	9.6	10.2	-	36.4
4	None	0.259	1.5	7.3	3.0	30.0
	400µg(4x100µg)	0.264	1.5	9.5	6.1	38.8
5	None	0.230	5.7	8.7	2.8	21.3
	500µg(4x125µg)	0.242	7.0	11.0	4.1	27.3
6	None	0.677	-	8.3	2.4	15.0
	500µg(4x125µg)	0.682	-	11.8	4.1	20.2

TABLE XXI

EFFECT OF ANGIOTENSIN II AND CO-FACTORS ON RAT ADRENAL GLANDS IN VITRO

Incubation Medium	Adrenal Tissue (gm)	Steroid Hormones µg/gm/hr			
		18-OH-B	ALDO	18-OH-DOC	B
KRG	0.327	9.6	11.0	13.5	28.4
KRG + NAD + G-6-P	0.349	9.0	13.8	17.2	76.9
KRG + NAD + G-6-P + Angiotensin II 400 µg (4 x 100 µg)	0.331	10.4	18.9	19.0	98.5
KRG + NAD + G-6-P	0.564	8.4	12.3	--	58.5
KRG + NAD + G-6-P + Angiotensin II 200 µg (4 x 50 µg)	0.560	8.2	9.1	--	66.5

(100 µg every 30 minutes) resulted in a further increase in the secretion of aldosterone, 18-OH-B and corticosterone. A lower dose of angiotensin II (50 µg every 30 minutes) caused only a slight increase in the secretion of corticosterone.

The changes in the pressor activity of angiotensin II after the incubation with rat adrenal tissue have been investigated. The bio-assay of angiotensin II was kindly carried out in the laboratory of Dr. J. Genest, Hotel-Dieu Hospital, Montreal by Dr. R. Boucher.

Adrenal quarters (0.502 gm) were incubated in siliconized vessels with 20 ml KRG solution. At the beginning of the incubation, 100 µg of angiotensin II was added to the incubation medium. For bio-assay 1 ml of the incubation medium was withdrawn after 15 and 30 minutes.

Another dose (100 µg) of angiotensin was added to the incubation medium after 30 minutes and the incubation was continued for a total of 120 minutes. Samples for bio-assay were withdrawn after 60 and 120 minutes of incubation. Table XXII. No reduction of biological pressor activity was demonstrable during the first 60 minutes. A marked decrease in pressor activity took place after an incubation period of 120 minutes.

TABLE XXII

INACTIVATION OF ANGIOTENSIN II BY RAT ADRENAL TISSUE

Rat adrenal quarters 0.502 gm

KRG buffer 20 ml.

Time of Incubation	Angiotensin II	Pressor Activity of Angiotensin II in µg of Angiotensin
15 minutes	5 µg/ml	6 µg/ml
30 minutes	5 µg/ml	6 µg/ml
60 minutes	10.8 µg/ml	10 µg/ml
120 minutes	10.8 µg/ml	5 µg/ml
	Reference standard	
	200µg/ml	180 µg/ml

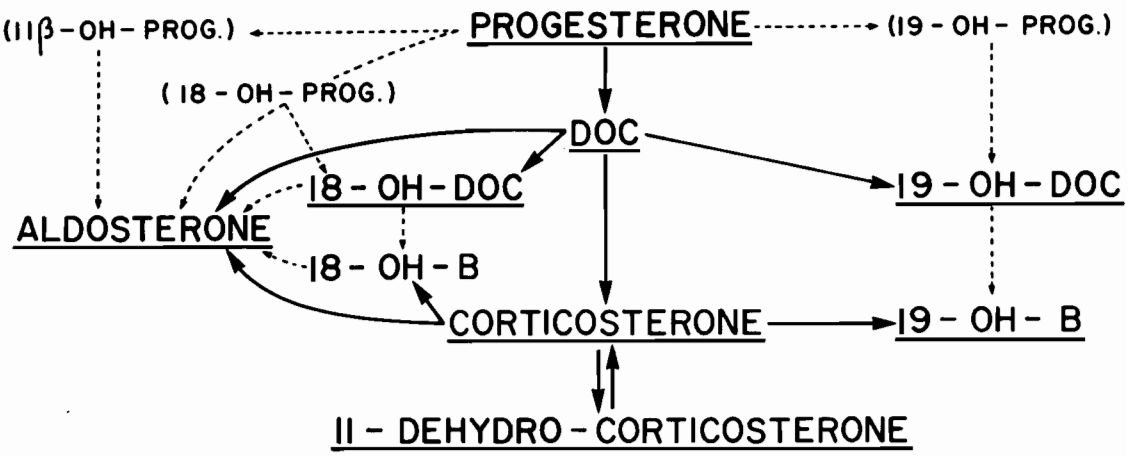

(11β-OH-PROG.)
PROGESTERONE
(19-OH-PROG.)
(18-OH-PROG.)
DOC
18-OH-DOC
19-OH-DOC
ALDOSTERONE
18-OH-B
CORTICOSTERONE
19-OH-B
11-DEHYDRO-CORTICOSTERONE

Figure 14. Biosynthetic pathways of steroid hormones in rat adrenal gland.

IV. DISCUSSION

Rat adrenal tissue in vitro is capable of converting progesterone to a number of products which by their chromatographic mobilities are more polar than progesterone. The following 8 steroid hormones have been characterized:- 18-OH-B, 19-OH-B, aldosterone, 19-OH-DOC, 18-OH-DOC, corticosterone, compound A and DOC. Using DOC-4-^{14}C as a precursor the same corticosteroids as mentioned above are obtained. As with progesterone-4-^{14}C, DOC-4-^{14}C is also converted to several unidentified substances. These have the same chromatographic mobilities as those derived from the progesterone precursor. The yield of identified steroids from DOC precursor is greater than that from progesterone, suggesting that DOC is an important intermediate in the biosynthesis of corticosteroids from progesterone. These observations are in accord with the findings of other authors (89, 18, 140). A proposed outline of the biosynthetic transformation of progesterone by the rat adrenal gland is shown in Figure 14.

The formation of 11β-OH-prog. and 18-OH-prog. from progesterone could not be demonstrated in this tissue although it has been suggested by Grant (140) that 11β-OH-prog. can serve as a precursor for aldosterone. Non-labelled 18-OH-prog. incubated with rat adrenals increases the yield

have suggested that 19-OH-S may be involved in water and electrolyte metabolism in the mongolian gerbil.

The 19-hydroxylation of C_{21} steroids is not limited to rodents and it has been demonstrated that adrenal glands from ruminants like ox, sheep and goat form 19-OH-DOC from DOC precursor (53). Adrenal homogenates of human fetal adrenals form 19-OH-DOC from DOC; this activity, however, was not demonstrable with adult human adrenal tissue (53). The 19 hydroxylase can be inhibited by SU 4885 which has been regarded as a reversible inhibitor for the 11 β hydroxylase system (141). The mechanism of the action of SU 4885 on the 19 hydroxylase activity is not clear.

In the biotransformation of androgens to estrogens, 19 hydroxylation is of major importance, and the formation of 19 oxygenated testosterone or androstenedione is an intermediate step for the aromatization of the ring A of the steroid nucleus (58). A search for estrogens as conversion products from progesterone or DOC in rat adrenals has remained unsuccessful. In studies with the steroid hormone aromatizing enzyme from microsomes of human placenta an aromatization of C_{21} steroids can not be demonstrated (57) nor could the conversion of progesterone by rat adrenal tissue to 3 β-hydroxy-19-nor-pregn-1,3,5(10)triene-20-one.

An extensive search in rat adrenal tissue for 17α-hydroxylated steroids as conversion products from proges-

terone have failed to reveal the presence of cortisol or 17α-OH-prog. Recent studies, however, show that 17α hydroxylase is demonstrable in young mice and in mongolian gerbils (47, 55). Experimental work with adrenal enzyme systems have shown that 17α hydroxylase has an optimal pH of 8.5 and the C-17, 20 cleavage enzyme requires pH close to 7.0 (142). The need for oxygen and NADPH has been demonstrated for both enzymes (142). Rat adrenal glands incubated in buffered KRG at pH 8.5 in presence of NADP converted progesterone in a similar pattern as in normal KRG solution at pH 7.4. The alkalinity of the incubation medium apparently failed to challenge the 17α hydroxylase activity and did not effect appreciably the total conversion of progesterone-4-^{14}C. Also, the presence of testosterone and 17 ketosteroids as conversion products from progesterone could not be demonstrated in the present study. Nevertheless, Ryan et al (63) have reported the presence of Zimmermann reactive substances in the urine of castrated male rats. These chromogens increase after ACTH treatment. The nature of these excretion products, presumably 17 ketosteroids, remains unknown. Hayano (143) and also Dorfman (64) have pointed out that the formation of C_{19} steroids from progesterone or pregnenolone can take place without an intermediary 17α hydroxylation. The possibility that C_{19} steroids of the rat are formed from C_{21} steroids outside

the adrenal cortex can not be excluded.

The present studies reveal that there is an exchange of labelled substances between the tissue and the incubation medium.

During the first 15 minutes of incubation the tissue accumulates a high proportion of the radioactivity which progressively declines on longer incubation. The conversion of progesterone by the quartered adrenals is so rapid that within 15 minutes more than one half of the added precursor is transformed; and most of the transformation products appear in the incubation medium. The sequence of events taking place during the incubation indicates that there is a continuous uptake of the precursor by the tissue, biotransformation of the precursor and a release of the products into the incubation medium. Factors governing the transport of the progesterone across the cell membrane and the exchange of synthesized products with the incubation medium have not been specifically studied.

In timed incubations it has been demonstrated that with adrenal quarters from normal rats corticosterone is always the principal product formed from progesterone. Other steroids appear in smaller quantities. The formation of corticosterone, 18-OH-DOC and DOC from progesterone increases rapidly within one hour of incubation. Aldosterone, 18-OH-B, 19-OH-DOC and compound A increase almost linearly

with the time of incubation. The velocity of steroid hormone formation from a precursor by adrenal tissue depends on the activity of enzyme systems and on the availability of precursor. Incubation of adrenal tissue for 15 minutes with progesterone-4-^{14}C gives rise to steroids which can be further hydroxylated like corticosterone, DOC, 18-OH-DOC. In the in vitro system these products may give rise to 18-OH-B and aldosterone. It is possible that the linear increase of 18-OH-B and aldosterone with the time of incubation is due not only to the direct utilization of progesterone but also to the use of already formed steroids present in the incubation system. A similar state of affairs can take place with the 19 hydroxylated steroids.

On functional zonation it has been shown that endogenous corticosterone is formed throughout the adrenal, whereas 18-OH-B and aldosterone originate from the zona glomerulosa. The distribution of 19 hydroxylase is not known. Biosynthesis of corticosterone from progesterone involves hydroxylation at C-21 and C-11. Comparison of the pattern of progesterone metabolism in the adrenals of normal rats and hypophysectomized rats reveals certain differences. Formation of corticosterone by adrenal tissue from hypophysectomized rats is markedly reduced. Histological studies have shown that atrophic changes in the cells of zona

fasciculata and reticularis take place rapidly after hypophysectomy. These zones in the adrenal are responsible for the greatest contribution toward the formation of corticosterone (11, 14, 21). The reason for the reduced biosynthesis of corticosterone after hypophysectomy is apparently due to decreased enzyme activity of the cells from the fasciculata and reticularis. The total formation of aldosterone and DOC in this adrenal preparation exceeds the biosynthesis of corticosterone, indicating that after hypophysectomy the enzyme systems for the formation of mineralocorticoids essential for maintenance of electrolyte balance are well preserved for some time.

Grant (140) has suggested that the adrenal cortex contains two 11β hydroxylase systems:- one which is distributed throughout the adrenal cortex and another which is limited to the zona glomerulosa. The latter is not inhibited by SU 4885. The presence of dual 11β hydroxylase systems in the zona glomerulosa may be responsible for the good yield of 18-OH-B and aldosterone from progesterone in the adrenal of the hypophysectomized rat. The 18 hydroxylase and 18 oxidase activity is also well preserved in rat adrenal glands after hypophysectomies.

In a single experiment, growth hormone was administered to hypophysectomized rats in vivo, a slight increase in average adrenal weight was observed. These adrenal glands showed also a slight increase in the conversion products

derived from progesterone. The isolated observation does not allow to draw any conclusions.

Studies with synthetic angiotensin II in vitro failed to show any increase in the conversion of progesterone to corticosteroids. The endogenous secretion of steroid hormones measured in vitro was increased when very high doses of angiotensin II were added. At these levels, this effect is of a doubtful physiological significance. A selective effect on aldosterone secretion by angiotensin II could not be demonstrated in these studies. Kaplan and Bartter (107) in their studies have shown that incubation of rat adrenal tissue with small doses of angiotensin II (5 µg/100 mg tissue) fails to alter the secretion of aldosterone and corticosterone. Comparable dose of angiotensin II however stimulates the formation of aldosterone in beef adrenal outer slices in presence of exogenous cholesterol (107). Angiotensin II administered intravenously to rats in doses of 40 to 50 µg/100 gm body weight produces a significant increase in the capacity of the adrenal glands to synthesize aldosterone with a minor effect on corticosterone (33). Singer et al (32) using hypophysectomized rats, showed an increased secretion of aldosterone in adrenal venous blood after infusion of angiotensin II. The change in aldosterone production was not clearly related

to the dose of angiotensin or the increase in blood pressure resulting from the infusion. Discrepancies between the stimulating action of angiotensin II on the secretion of aldosterone in vivo and the failure of a selective stimulation in vitro remains obscure. It has been shown that rat adrenal tissue does inactivate the pressor activity of angiotensin II on incubation, suggesting the presence of angiotensinase. In order to overcome the inactivation, angiotensin II was added repeatedly during the incubation. It is possible that for the action of angiotensin II on the zona glomerulosa of rats are necessary some other unknown factors which are present in vivo but are absent in vitro.

V. SUMMARY

Adrenal tissue from hooded adult male rats in vitro transforms progesterone-4-^{14}C to at least 23 labelled products. Of these, 18-hydroxycorticosterone, 19-hydroxy-corticosterone, aldosterone, 19-hydroxy-11-deoxy-cortico-sterone, 18-hydroxy-11-deoxy-corticosterone, corticosterone, 11-dehydro-corticosterone and 11-deoxy-corticosterone were characterized. These steroids are also formed from DOC-4-^{14}C. This is the first time that the presence of an enzyme system for hydroxylation at C-19 of the steroid molecule has been demonstrated in the rat adrenal. Attempts to show the formation of 17α-hydroxylated steroids, C_{19} steroids and C_{18} steroids from radioactive progesterone or DOC have failed. Timed incubations of rat adrenal tissue with progesterone-4-^{14}C show that more than one half of the precursor is metabolized after 15 minutes of incubation and the trans-formation continues at a slower rate on longer incubation. At all times of incubation, corticosterone is the principal conversion product in the incubation medium and within the tissue. Analysis of labelled products after various periods of incubation indicate that the tissue in vitro is contin-uously taking up the precursor, metabolizing it and releas-ing the formed products back into the incubation medium.

The total formation of mineralocorticoids, aldosterone

and DOC, by adrenal glands from hypophysectomized rats from progesterone-4-^{14}C is greater than the formation of corticosterone.

Synthetic angiotensin II fails to influence the conversion of progesterone-4-^{14}C in normal adrenal glands. The endogenous secretion of steroid hormones by such glands is slightly increased with high doses of angiotensin II.

A method of serial paper chromatographic separation of steroid hormones has been described.

The applicability of the Mattox Porter-Silber reaction for the chemical assay of corticosterone, aldosterone, 18-OH-DOC and 18-OH-B has been investigated.

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