FACTORS INFLUENCING RAT ADRENAL STEROID HYDROXYLATIONS *IN VITRO*

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FACTORS INFLUENCING HYDROXYLATIONS OF LABELED STEROID PRECURSORS BY DIFFERENT RAT ADRENAL PREPARATIONS

(Abstract)

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The effects of the heterogeneity of rat adrenal tissues of different preparations, and of other controllable factors on the corticosteroidogenesis from labeled steroid precursors were studied. A double isotope method for the simultaneous measurement of the specific activities and internal productions of steroids was used.

Evidence points to a deficiency of 21-hydroxylase activity relative to the 11β-hydroxylase in the PIM (preincubation medium), and in the glomerulosa when NADPH was added. The NADPH concentrations seemed to be important in the imbalance of the different hydroxylations - smaller concentrations promoting only the 21-hydroxylation, intermediate the 21- and 18-, but especially 11β-hydroxylation, and higher being relatively inhibitory to the three. Different pools of enzymes and precursors were demonstrated in slice incubations.

The glomerulosa mitochondria had a very low

hydroxylation activity, but 18-hydroxylation of corticosterone occurred readily. DOC was inhibitory to the 11β-hydroxylation of glomerulosa, but not fasciculata mitochondria.

The interpretation of the in vitro data obtained was discussed.

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By

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To my

d e a r

PARENTS

and

t r u e

FRIENDS

for their

LOVE, UNDERSTANDING and KINDNESS

In Loving Memory

of my

THREE BELOVED SISTERS

D E D I C A T E D

to

Dr. John S. L. Browne

B.A.(McG), B.Sc.(McG), M.D.,C.M.(McG),
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Professor and Chairman

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(1955 - 1969)

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who

Introduced Me To Endocrinology

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LEGENDS TO THE ABBREVIATIONS OR TRIVIAL NAMES USED

ACTH	adrenocorticotropic hormone
ATP	adenosine-5'-triphosphate
b.p.	boiling point(s)
c, mc, uc	curie, millicurie, microcurie
c.p.m.	counts per minute
d.p.m.	disintegrations per minute
Δ^4	double bond between C-4 and C-5 of the steroid nucleus
Δ^5	double bond between C-5 and C-6 of the steroid nucleus
G-6-P	glucose-6-phosphate
G-6-P-D	glucose-6-phosphate dehydrogenase
gm.,mg.,µg.	gram(s), milligram(s), microgram(s)
g	acceleration due to gravity
KRBG	Krebs-Ringer Bicarbonate Glucose solution
max.	maximum
ml.	millilitre
min.	minute
M, mM	molar, mole; millimolar, millimole
µmole	micromole
mµmole	millimicromole (10^{-9} mole)
mµ	millimicron (10^{-9} metre)
NAD	nicotinamide adenine dinucleotide
NADH	" " " (reduced form)
NADP	" " " phosphate
NADPH	" " " " (reduced form)

LEGEND TO THE ABBREVIATIONS OF TRIVIAL NAMES USED (Cont'd.)

oxid.	oxidation, oxidized
PC	paper chromatography
Pi	inorganic phosphate
PIM	preincubation media
S. A.	specific activities (d.p.m./mμmole)
TCA	tricarboxylic acid cycle
TLC	thin-layer chromatography
T.P.G.	toluene propylene glycol
v/v	volume/volume
x	multiply, times
x-ray	Roentgen rays

NOMENCLATURE OF STEROIDS MENTIONED IN THIS THESIS

Trivial or Abbreviated Names	Systematic Names
Aldosterone* (Aldo.)	11β,21-Dihydroxy-pregn-4-ene-3:20-dione-18-al
ASD (androstenedione)	Androst-4-ene-3:17-dione
Cholesterol	Cholest-5-ene-3β-ol
Corticosterone (Compound B)	11β,21-Dihydroxy-pregn-4-ene-3:20-dione
11-Dehydrocorticosterone (Compound A)	21-Hydroxy-pregn-4-ene-3:11:20-trione
11-Deoxycorticosterone (DOC)	21-Hydroxy-pregn-4-ene-3:20-dione
18-Hydroxycorticosterone* (18-OH-B)	11β:18:21-Trihydroxy-pregn-4-ene-3:20-dione
18-Hydroxy,11-deoxy-corticosterone * (18-OH-DOC)	18,21-Dihydroxy-pregn-4-ene-3:20-dione
Δ^5-Pregnenolone	3β-Hydroxy-pregn-5-en-20-one
Progesterone (Prog. or P)	Pregn-4-ene-3:20-dione
Progesterone-20β-ol (Prog. reduced at C-20)	20β-Hydroxy-pregn-4-en-3-one
11β-Hydroxyprogesterone (11β-OH-P)	11β-Hydroxy-pregn-4-ene-3:20-dione

* open form

CHAPTER I

PURPOSE OF THE INVESTIGATION

It has been customary to report the results of the studies of the steroid biosynthetic pathways in terms of per cent conversions of a labeled precursor to its different products. Although this approach has been commonly applied and has furnished valuable information, it assumes that the labeled precursor would be metabolized in a manner identical to the endogenous precursor(s) available within the system under study.

However, there is increasing evidence that different pools of precursors, cells and enzymes are artificially produced in an incubation. For example, it had been shown that the preincubation media (PIM) (1 , 2) possess some enzymatic activities mostly of the 11β-, 18- and 21-hydroxylases, which are associated with cellular particles and leaked from the cells (3). The 21-hydroxylase in a dilute medium is inactivated much more readily than the 11β-hydroxylase, resulting in an accumulation of 11β-hydroxyprogesterone in a dilute medium (4). Moreover, there are different histological zones in the mammalian adrenal cortex which are the sites of the production of different types of

steroid hormones. It has also been reported that there are two dissimilar types of cells present in adrenal sections (4a). All these phenomena point to the heterogeneity of an in vitro system. Thus a given precursor can be metabolized in a different manner when incubated with either the media (of preincubation or incubation) or the adrenal tissues (the glomerulosa and the fasciculata tissues separately or together), justifying the presence of different pools of enzymes with different reactivities. Negligence of this concept would lead to the erroneous interpretation of the results.

Hence, the different pools of enzymes had to be studied systematically. In order to tackle part of the problem, a method has been developed by Dr. J. STACHENKO of this Laboratory for the simultaneous measurement of the production of steroid from the endogenous precursor as well as the labeled precursor added to the system. It also gives the final specific activity of the labeled precursor.

Applying this method of measurement, the purpose of this Thesis was to study the effects of some intrinsic and controllable factors on the biotransformation of an added precursor in vitro. These factors were: the level of organization of the different zones of the adrenal cortex,

from the sub-cellular level to the intact gland, the duration of the incubation, the concentrations of precursor, enzymes and cofactors (malate, NADP and G-6-P).

It was hoped that knowledge derived from such studies would help to clarify, at least in part, the underlying mechanisms of the rat adrenocorticoid biosynthesis.

CHAPTER II

REVIEW OF LITERATURE

1. Introduction.

The objectives of the investigation reported in this Thesis are to demonstrate the heterogeneity of the steroidogenic responses by different rat adrenal tissue preparations, and the different factors which are influential in the adrenocorticoid biosynthesis in vitro.

Accordingly, this review attempts to give a concise account of the in vitro adrenocorticoid biosynthesis, with respect to the experimental methods commonly used and the available data. Emphasis will be given to the results obtained with the rat adrenal cortex, which was studied in this Thesis because of the ease of its separation into different histological zones (5).

2. The Adrenal (Suprarenal) Gland.

The mammalian adrenal glands had been identified and described as early as the middle of the sixteenth century (6). Its vital importance had been ascertained by THOMAS ADDISON's studies on adrenal insufficiency (Addison's disease) in 1855 (7), as well as the investigation of BROWN-SEQUARD (8) on the effect of adrenalectomy.

The suprarenal glands are paired, flattened, yellow masses of tissue that lie, as their name implies, in contact with the upper poles of the kidneys. Anatomically, the adrenal gland was shown by KOELLIKER (9) to consist of two distinct parts, the cortex and the medulla. These two parts have different embryological origins, histological characteristics, and physiological functions (10).

Embryologically, the adrenal cortical tissue is a mesoderm structure derived from the mesonephros. ARNOLD (11) had further classified the so called adrenal cortex according to its layout into: the outermost part or zona glomerulosa (Z. G.), bound by the capsule, the middle part or zona fasciculata (Z. F.), and the innermost part or zona reticularis (Z. R.), adjacent to the medulla. Microscopically, the rat Z. G. consists of parenchymal cells grouped into little, irregular clusters with capillaries in between them. These cells tend to be columnar, with smaller and darker nuclei than those of Z. F.; their cytoplasm is of a more even texture, and contains lipid droplets. The Z. F. is a thick layer in which the cells are arranged in fairly straight cords, which run at right angles to the surface and have straight capillaries between them. The cells are roughly polyhedral and, in preparation, their cytoplasm appears to be extensively vacuolated, due to their large number of lipid droplets and are sometimes termed spongiocytes.

Cholesterol is more concentrated in these cells than in any other part of the body (HAM et al. (10)). The Z. R. is a relatively thin layer in which the cells are disposed in cords which run in various directions and anastomose with one another. Sinusoidal capillaries occupy the interstices between the cords. The Z. R. cells vary in appearance. Some have small dark nuclei and acidophilic cytoplasm and appear to be degenerating. Others have lighter nuclei and cytoplasm. Some cells may have considerable amounts of pigment (10). Between the Z. G. and Z. F. in the rat is a zone called transition or soudanophole. This zone is very sensitive to ACTH in vivo and mitosis is active there.

The steroid secreting endocrine cells are very similar to one another in their ultrastructural features. They possess: (i) a remarkably extensive smooth-surfaced endoplasmic reticulum, (ii) a very prominent Golgi complex, (iii) mitochondria of highly variable size and often of unusual internal structure, (iv) numerous lysosomes and a tendency to accumulate lipochrome pigment, and (v) the presence of lipid droplets in greater or lesser numbers (FAWCETT et al. (12)). The mitochondria in the Z. G. of most species are elongate and have lamellar cristae oriented perpendicular to the long axis of the organelle. In the Z. F. and Z. R., they are short rods or spheres (12).

3. Experimental Approach to the Study of Adrenal Steroidogenesis.

The indispensability of the adrenals for life (13) and the dramatic therapeutic effects (14) of some adrenocorticoids (e.g., cortisone) had triggered tremendous research on the steroidogenesis over the last decades. Various types of investigation had been devised, both in vivo and in vitro. Comprehensive reviews have appeared dealing with the numerous aspects of the processes involved in the studies of the various cortical secretory products under different experimental conditions (15,16,17,18).

The experimental approach to the study of adrenal corticoid metabolism has involved experiments in which the "disturbances" have ranged from the minute amounts of an isotopically labeled substance administered to the diet of the intact experimental animal, to the manipulations by which the animal is finally sacrificed, with its organ(s) removed and the enzyme(s) isolated from the organ(s).

(A) The in vivo method

The physiological studies involve the analyses of the urinary excretion of steroids and their metabolites, after the administration of steroid or other substances which would influence the steroidal metabolism. These studies have

provided valuable information which has formed the basis of our present understanding of corticosteroid metabolism in man.

For the direct study of the adrenocorticoid biogenesis in the animal, VOGT in 1943 (19) had evolved a method using direct blood sampling from the rat adrenal vein by cannulation. However, despite the "near physiological" conditions employed, the method suffers from the limitations of requiring much technical skill and the probable "stressful" conditions involved. Moreover, the trace corticosteroids may escape the analytical detection.

More recently, MCDONALD et al. (20) had succeeded in transplanting one adrenal gland to a combined carotid artery-jugular vein loop in the Merino-breed sheep. This group reported a normal adrenal steroid hormone secretion and histological examination of the transplanted adrenal long after the operation.

In fact, the numerous uncontrollable physiological and metabolic processes occurring in the intact animal have made it extremely difficult to obtain a true picture of the in vivo adrenocorticoid biogenesis.

(B) The in vitro method

Much of the work available on the pathways of steroid

biosynthesis has been done in vitro.

The birth of the in vitro method owed much to the observations of HARRISON (21) and CARREL (22) that animal tissues, and even the vital organs, can be kept "alive" under physiological conditions for a limited period of time after their surgical removal from the animal. In essence, the in vitro method is relatively easier and more controllable than the in vivo approach. It commonly employs excised glands either for incubation in a buffer system with or without precursor(s) or for perfusion, to determine the pathway of adrenocorticoid biogenesis. With the technological advancements in the productions as well as the measurements of radioactive isotopes, isotopically labeled steroids (or related substances) have been widely used as precursors or tracers in vitro.

However, the in vitro method only reveals reactions that may occur in vivo, with no certainty of any physiological importance, as HECHTER once remarked (23), nor the extent to which it does occur in vivo, since there is almost a total loss of regulatory mechanism and the artefacts are common.

(1) Perfused, isolated adrenal studies. In this method, the substrates in the perfusion media (either whole blood, plasma, or artificial isotonic media) are presented

through vascular channels to surviving enzyme systems present in the cells, and the steroid content of the perfusate analyzed.

Beef adrenal perfusions had been undertaken by HECHTER et al.(23,24) in 1948. Most of the pathways of the steroid biogenesis in the beef adrenal have been elucidated by the perfusion method.

Admittedly, the best of the perfusion conditions cannot exactly duplicate the in vivo physiological conditions. The animal, whose adrenal is being prepared for perfusion, undoubtedly releases ACTH from its pituitary and the adrenals are stimulated. There is also a mechanical filtration of the perfused substrate, leading to the progressive blockade of the vascular system and the reduction of the substrate available for experimentation (23).

(ii) Adrenal slice incubations. Tissue slices represent an organized surviving tissue, the metabolism of which reflects that of the original tissue. This method, developed by WARBURG et al. (25), allows for the controlled variations in the suspending medium in addition to chemical analysis of the latter for changes in metabolite content.

It had been observed by SAFFRAN et al. (26) that rat adrenal slices produced steroids when incubated in saline

medium. This technique involved the excision of adrenal glands which are dissected free from the surrounding fat and either bisected or quartered. The glands can be obtained from normal or pretreated animals. The prepared glands are preincubated in a physiological medium. The preincubation greatly enhanced the sensitivity of the isolated adrenals to the factors they were to be exposed to in the incubation medium (SAFFRAN et al. (27)). The preincubated glands are reincubated under the required conditions in fresh physiological media, which are modeled after the extracellular fluid with its content of sodium chloride and calcium ions. Despite the deviations of the secretion rate from the normal within the cells of the slice, these preparations have proven to be extremely useful because of the simplicity of the operation.

This method has been used to study either the effect of a trophic factor (28) or an inhibitor (29) on the adrenocorticoid biogenesis, or the pathway of the biogenesis after the administration of a precursor.

The products of reaction accumulate in course of the incubation. They may either inhibit (30 - 32) the rate of the reaction, or may be further converted into secondary and tertiary products in a "normal" or "abnormal" pathway (24). Accordingly, SAFFRAN (33) and TAIT (34) had a devised a contin-

uous flow (superfusion) technique for such incubations. Characteristically, this method involves the constant removal of the metabolites formed with the simultaneous renewal of the incubation medium, and thus can maximally overcome the recirculation of the products formed.

The tissue slice technique, however, gives only the overall pictures of metabolism rather than concerning single reactions and enzymes (ELLIOTT (35)).

(iii) Adrenal tissue homogenate incubations. The homogenates represent the level of organization in which the cell membranes have been disrupted. Its use is based on the assumption that any reaction which occurs in living cells can occur in cell-free preparations under appropriate conditions. The general principles of this approach were due to POTTER and ELVEHJEM in 1936 (36). Tissue homogenates are used for the study of enzymic reactions either directly in the form of whole homogenate, or as the starting material for the preparation of particulate components of cells (37).

The adrenal tissues are homogenized in an all-glass, motor-driven or a POTTER-ELVEHJEM homogenizer, either in a cold KRBG medium, or in iso- (0.25 M) or hypertonic sucrose. Practically all the cells are broken and the subcellular components remain unbroken (38). Optimal duration of the homogenization may be doubled without affecting the results (37).

The incubation conditions of the homogenates, such as dilutions and temperature, have been found to be rather important in the interpretation of the in vitro steroidogenic pathways (TSANG (39)).

(iv) Sub-cellular fraction incubations. The cell fractionation technique has proven useful in the studies designed to determine the location of the metabolic processes within the cell, as it permits the separation of various particulate structure of the cells. With this technique, described by SCHNEIDER and HOGEBOOM (40), the homogenates are further subjected to differential ultracentrifugation at low temperatures (38) to yield sub-cellular particles and fractions sedimented at different speeds, e.g., nuclei, "light" and "heavy" mitochondria (HOGEBOOM et al. (41), CAMMER et al. (42), PERON et al. (43)), microsomes and supernatant fractions. The fractions are incubated in a cofactor-fortified isotonic medium.

(v) Purified enzyme incubations (44-47). The molecular details of regulatory mechanism operating at the level of catalysis have come from studies of isolated, purified enzymes. Reliable information concerning the kinetics, cofactors, active sites, structure, and mechanism of action also requires highly purified enzymes. Accordingly, various groups (48,49) had attempted to solubilize the adrenal cortex

hydroxylases.

SHARMA et al. (50) purified 11β-hydroxylase by homogenizing the mitochondrial preparations in distilled water, before subjecting it to ultrasonic vibrations followed by centrifugation at 105,000 g. The resulting clear supernatant fluid was dried by lyophilization (yielding 1 gm. dry enzyme per 50 gm. whole adrenal). The aim was to achieve the maximum specific activity (enzyme units per mg. protein) with the best possible recovery of initial activity.

The purified enzyme is incubated with its substrate under optimal conditions for characterization and requirement studies (48). Manometric technique (51) have been extensively used for such enzyme assays.

(C) Methods of characterization and measurement of adrenocorticoids

The difficulties in the isolation of the adrenocorticoids have been due to the large number of closely related compounds which need separations (52).

The incubates are extracted with polar solvents (either ether, chloroform, dichloromethane, or ethyl acetate, etc.). The dried extracts are subsequently purified and concentrated by column, paper, thin-layer or gas chromatography, or a combination of these.

The application of column chromatography (TSWETT, 1910) to steroid analyses owed much to its development by MARTIN and SYNGE (53) in 1941. Paper chromatography has been developed in the steroid field by ZAFFARONI et al. (54, 55), and later, SAVARD (56), BUSH (57) and EBERLEIN et al. (58). Thin-layer chromatography (TLC) is widely used for the separation and characterization of steroids (59) after STAHL (60) who standardized the procedure. Gas chromatography (61) has developed rapidly since MARTIN and JAMES described their first experiments in 1952. These methods have proven to be extraordinary tools capable of separating literally dozens of components, even in a sample of less than a microgram (gas chromatography). Further purification of the steroids from the chromatographic eluates involves the use of physical methods such as crystallization (62).

The identification of the steroid compound is effected through the characterization of its different functional groups by chemical methods. For example, the α,β-unsaturated ketone in ring A is tested by: (i) its U.V. absorption ($\lambda_{max.}$ 240 mμ (63)) (64), (ii) the isonicotinic acid hydrazide (INH) reaction (65) or (iii) the soda fluorescence (66) reaction. The 20-ketol side-chain can be characterized by: (i) the reduction of the tetrazolium salts (64, 65, 68), or (ii) oxidation with periodic acid (HIO_4) (69, 34). The 21-hydroxyl

group can be acetylated (76 - 78) and the acetates characterized by their chromatographic mobilities (Table III). The dihydroxyacetone side-chain can be detected by the formation of osazone ($\lambda_{max.}$ 410 mμ) in the PORTER-SILBER reaction (75), which is also given by the 18-hydroxy,11-dehydrocorticosterone and 18-hydroxy,11-deoxycorticosterone. The 11β-hydroxyl group can be oxidized by chromium trioxide (CrO_3) to the 11-keto function (78). In addition, sulfuric and phosphoric acids form different characteristic spectra (chromogen formation), specific for individual steroids (ZAFFARONI (79)).

The steroid identification is reinforced by physical approach which includes: (i) mass spectrometry (80, 52), (ii) melting points, (iii) optical rotation (81), (iv) infrared absorption spectroscopy (82 - 84), (v) nuclear magnetic resonance (NMR) (85, 86) or nuclear spin resonance spectroscopy (NSR), and (vi) X-ray crystallography (87 - 89) which is very useful for the characterization of the steroid molecule in detail.

Some of the methods outlined above have been developed into quantitative methods for the spectrophotometric measurements of the light absorption at certain wavelengths (90).

The availability of liquid scintillation spectrometers for counting weak beta-emitting isotopes has extended the use of tritium and carbon-14 as valuable tools for the biochemical and analytical studies (91,92). Accordingly, the use of these isotopes have been designed in various ways, such as isotope dilution analyses (93) and the double isotope derivative methods (94,90,95). Such isotopic methods are independent of the losses incurred during the extensive purification processes of the steroids.

4. Experimental Data of the Study of Adrenocorticoid Biogenesis.

(A) Steroids produced by adrenal cortex in vitro

Some twenty-nine different steroids were isolated from the bovine adrenal tissue extracts (cortin) by the early investigators (PFIFFNER et al. (96), KENDALL et al. (97,98) and REICHSTEIN et al. (99)). With the exception of the estrogens and of the sterols, all these steroids have either nineteen or twenty-one carbon atoms as well as the characteristic cyclopentanoperhydrophenanthrene nucleus. A few of them are biologically active in carbohydrate (glucocorticoids) or mineral (mineralocorticoids) metabolism.

In all species studied, cortisol and corticosterone, or a mixture of both, are quantitatively and qualitatively the most important glucocorticoids synthesized or released

in vitro by the mammalian adrenal cortex (HAYANO et al. (100)). Singly or together these two compounds may contribute up to eighty-five per cent or more of the total Δ^4-3-keto-steroids, according to BUSH (101). Rat (Order Rodentia), placed in Group II (SANDOR (102)) of the tentative classification of animals according to the chemical nature of the corticoid hormones elaborated, secretes only 17-deoxycorticosteroids. Corticosterone is the major secretory product in the rat (5 ,103,104,105,106). Aldosterone, first isolated by SIMPSON and TAIT in 1952 (107), is the most potent mineralocorticoid produced. It is however, secreted in minute quantities (estimated 25 - 103 DOCA eqts./Kg/hr. (66)) by intact rats under normal conditions.

The second most abundant corticosteroid produced in vitro by the rat adrenal had been reported (BIRMINGHAM et al. (108) and PERON (109,110)) to be 18-hydroxy, 11-deoxycorticosterone (18-OH-DOC). The isolation of 18-hydroxycorticosterone (18-OH-B) in the rat had been made by PERON (109,110) and in bovine and human glomerulosa by SANDOR and LANTHIER (111). It was shown to be produced by the zona glomerulosa of the rat adrenal cortex by STACHENKO et al. (5).

Labeled 19-hydroxy, 11-deoxycorticosterone had been isolated and 19-hydroxycorticosterone tentatively identified by LUCIS et al. (112) in incubations with ^{14}C-labeled

progesterone or 11-deoxycorticosterone. The structural formulae of the major steroids produced by the rat adrenals studied in this Thesis are given in APPENDIX A.

(B) Pathways of adrenocorticoid biogenesis

The pattern of steroid synthesis in adrenal cortex is complex both in terms of the multiplicity of reactions involved and the structural and spatial organization of these reactions within the cell.

The sequence of the enzymatic reactions leading to the formation of active corticosteroids by the adrenal cortex had been indicated by the perfusion studies of HECHTER et al. (113) and HEARD et al. (114). According to these authors, the possible routes of adrenal corticosteroid biosynthesis is through acetate, involving cholesterol and Δ^5-pregnenolone and progesterone as key intermediates.

(i) From acetate to cholesterol. SRERE et al. (115), ZAFFARONI et al. (116), HAYNES et al. (117), and HECHTER et al. (118) demonstrated that the biosynthesis of adrenal corticosteroids started from acetate. Cholesterol is the key intermediate (119,120,121,118,119,113,115,116,122,123-125) but may not be an obligatory one (114,122,126). Most of the sequences of the enzymatic reactions involved are still obscure and are presumed to be similar to the pathway elucidated in the

liver by BLOCH (127). From the work of this author, HECHTER et al. (23,113,128,129) has established the schematic presentation of the pathway in the adrenal cortex as shown in Figure 1.

(ii) From cholesterol to Δ^5-pregnenolone. The work of ZAFFARONI (129) and CASPI et al. (130) strongly support the view that corticosteroids are synthesized via cholesterol. Cortical preparations cleave cholesterol into Δ^5-pregnenolone and isocaproic acid (131,132). The basic mechanism for cholesterol side-chain cleavage is associated with the initial hydroxylations at C-20 and C-22 (133), with the flow of electrons and oxygen activation similar to those of the steroid 11β-hydroxylations proposed by OMURA et al. (134,135). The scheme of several pathways leading to the formation of Δ^5-pregnenolone from cholesterol or cholesterol sulfate (136, 137) is given in Figure 2.

(iii) From Δ^5-pregnenolone to progesterone. The biotransformation of Δ^5-pregnenolone to progesterone by adrenal cortex was established by HECHTER et al. (23), LEVY et al. (138) and SAMUELS et al. (139,140). It involves two enzymatic steps (TALALAY (141), EWALD et al. (142) and KRUSKEMPER et al. (143)): the oxidation of the Δ^5-3β-hydroxy function to the 3-ketone, followed by the shifting of C-5, C-6 double bond to C-4, C-5 bond. The enzymes and co-enzyme

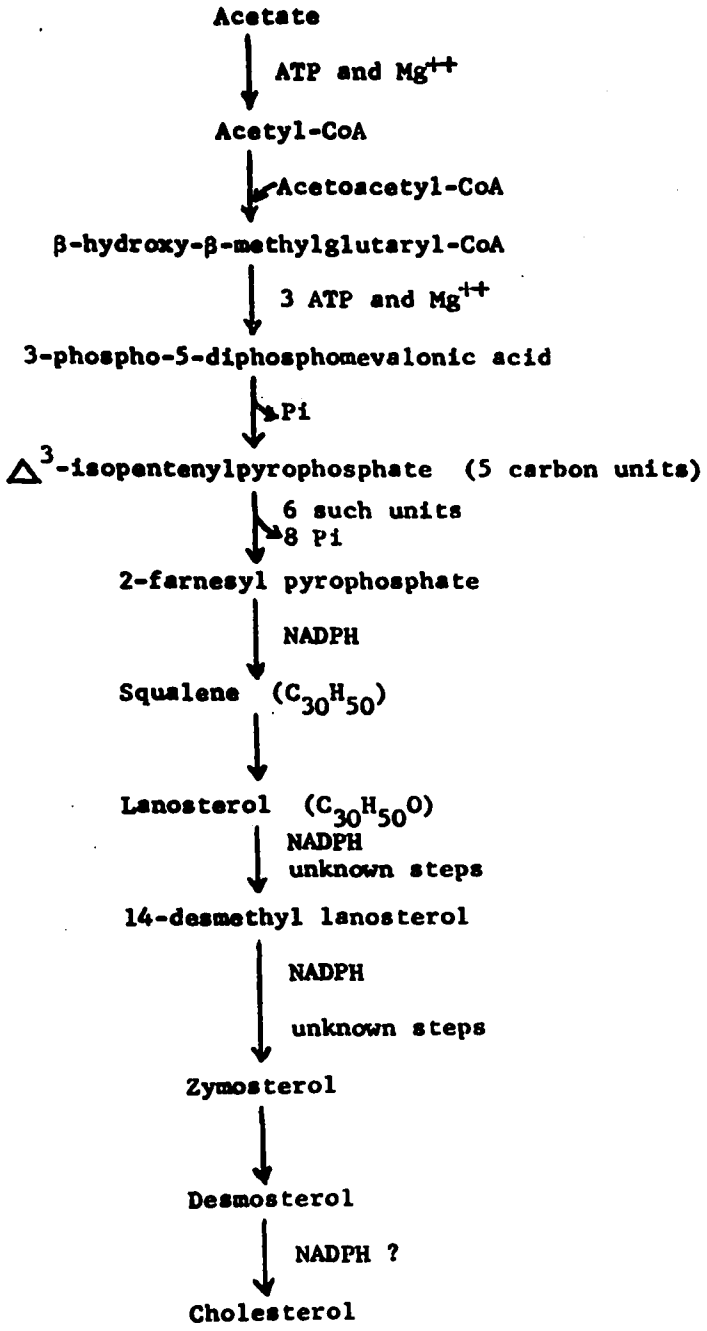


Fig. 1. Biosynthesis of cholesterol from acetate.

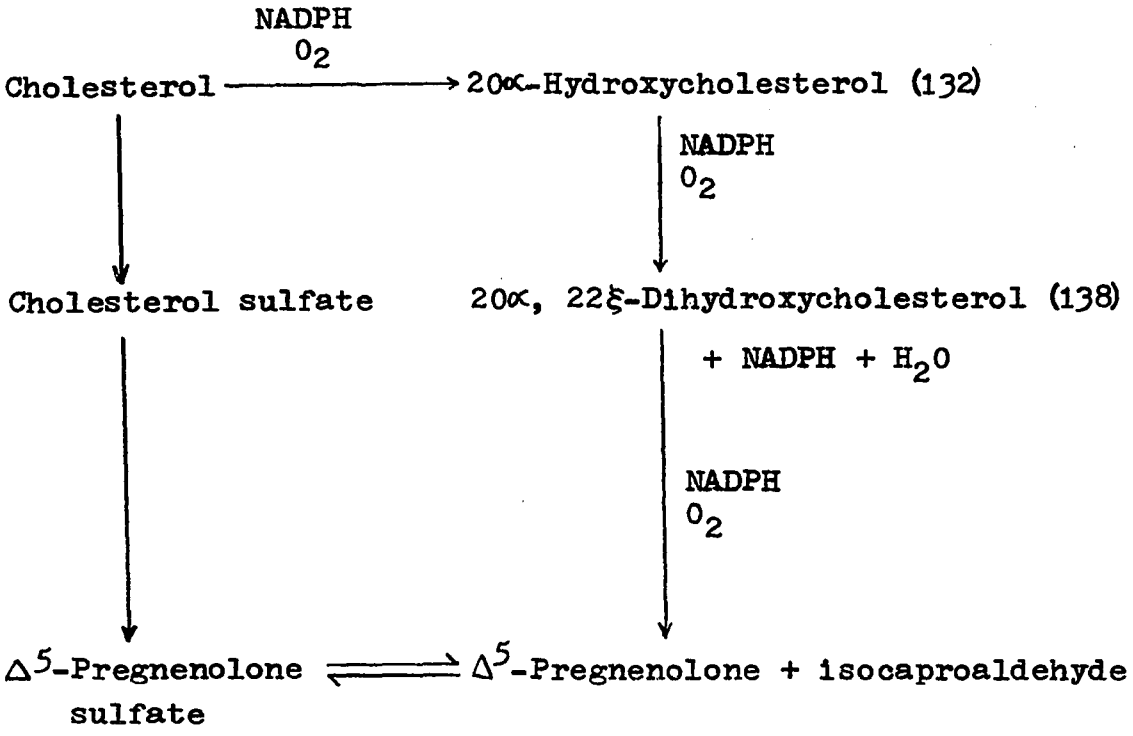


Fig. 2. Biosynthesis of Δ^5-pregnenolone from cholesterol (partially adapted from SHIMIZU (157)).

involved had been described by SAMUELS et al. (140), TALALAY et al. (144), KAWARHARA et al. (145) and KOWAL et al. (146,147)). Progesterone and NADH inhibit these reactions (39).

(iv) From progesterone to corticosterone. Progesterone is readily converted by rat adrenal to corticosterone, with 11-deoxycorticosterone as intermediate (EICHHORN et al. (148), LUCIS et al. (112) and HECHTER et al. (128)). DORFMAN et al. (156) demonstrated that 11β-hydroxylation is most efficient when Δ^4-3-ketonic ketols were substrates. These findings suggested that hydroxylation at C-21 must precede the C-11β-hydroxylation.

Progesterone gives rise to 11β-hydroxyprogesterone in course of adrenal corticosteroid biosynthesis, mostly in homogenate and perfusion experiments (HECHTER et al. (149)). There are still discussions whether 11β-hydroxyprogesterone is a precursor of corticosterone (BROWNIE et al. (150), LEVY et al. (138), EICHHORN et al. (148), GIROUD and STACHENKO (151, 152), KRAULIS and BIRMINGHAM (153), MAKOFF et al. (154) and CREANGE et al. (155)). 11β-hydroxylation does not occur with C-21-deoxysteroids like progesterone or 17-hydroxyprogesterone when a purified mitochondrial system is used (149).

There is increasing evidence supporting the view that progesterone is not necessarily an obligatory intermediate

in corticoid biosynthesis (WELIKY et al. (158), BERLINER et al. (159), PASQUALINI et al. (160), KOWAL et al. (147)). Steroid hydroxylases can act directly on Δ^5-pregnenolone (147, 158 - 160) by having dehydrogenation and isomerization occurring after the hydroxylations (KOWAL et al. (147), WHITEHOUSE et al. (161)).

(v) Biosynthesis of aldosterone. Labeled progesterone, DOC and corticosterone give rise to radioactive aldosterone (162 - 166), which is produced by the zona glomerulosa cells of the mammalian adrenal cortex (GIROUD et al. (5), AYRES et al. (167,168)). TAIT et al. (169) calculated that at least 50% of the aldosterone produced was derived from corticosterone. KAHNT and NEHER (170) have stressed that corticosterone lies on the main pathway of aldosterone biosynthesis. AYRES et al. (162) and RAMAN et al. (171) favoured the view that the pathway from progesterone leading to DOC and corticosterone is likely the major pathway for aldosterone biosynthesis. As suggested by KRAULIS et al. (172) and NICOLA et al. (173), possible alternative pathways via 18-hydroxy or oxo-derivative of pregnenolone may occur.

NEHER et al. (174) and ULICK et al. (175) and RAMAN et al. (49) have pointed to the importance of the 18-hydroxylated steroids as possible intermediates in the biosynthesis of aldosterone. All aldosterone producing species can synthesize

aldosterone from corticosterone concomitant with the production of 18-hydroxycorticosterone (SANDOR (102)). Although 18-hydroxycorticosterone may be the intermediate and obligatory precursor of aldosterone in vertebrate adrenals, exogenous 18-hydroxylated substances are not efficiently incorporated (NICOLIS and ULICK (111, 176)), probably due to their 18-20 cyclic hemiketal structures (STACHENKO et al. (177) and ULICK et al. (176)) in solution, whereas the open form may be the actual intermediate in aldosterone biosynthesis (178). However, PASQUALINI (179) observed significant incorporation of radioactive 18-hydroxy-corticosterone into aldosterone in human adrenal tissues, both normal and with tumor.. STACHENKO et al. (152) reported the concomitant inhibition of 18-hydroxycorticosterone and aldo-sterone production from corticosterone by metopirone (SU-4885) and suggested that 18-hydroxylation of corticosterone (or a closely related steroid) might be a necessary step in aldo-sterone biosynthesis.

RAMAN et al. (49) noted that the presence of $NADP^+$ or NAD^+ in the incubation media resulted in the conversion of 18-hydroxycorticosterone into 18-hydroxy,11-dehydrocorticosterone and not aldosterone. The 18-hydroxy,11-dehydrocorticosterone was not found to be a precursor of aldosterone in the in vitro system (49). The formation of 18-hydroxy,11-dehydrocorticosterone

and the inhibitory effect of 18-hydroxycorticosterone (but not 18-hydroxy,11-deoxycorticosterone or aldosterone) on the 18-hydroxylation of corticosterone appears to be of significance in the control of aldosterone biosynthesis (49).

(C) Adrenal cortex hydroxylases (184)

Many of the enzyme systems involved in the corticosteroid biosynthesis (139, 156) are hydroxylases. They have been classified by the International Commission on Enzymes as sub-roup EC-1-99-1 of the main class oxido-reductase. They all have some common characteristics with respect to their requirements for activities. Only the 21-, 11β- and 18-hydroxylases of the corticosteroids, relevant to this Thesis, are presented here.

(a) 11β-hydroxylase (EC 1.99.1.7) This is the most intensively studied steroid hydroxylase, especially by HAYANO et al. (191, 30, 192, 15). As first reported by SWEAT (193), it is found in the mitochondrial fraction (100, 194, 192, 193, 195, 150, 196, 197). It has an optimum pH of 7.4 (HAYANO and DORFMAN (30)). The 11β-hydroxylase system has an optimum temperature of 37°C. and its rapid destruction takes place at 41°C. or above.

GRANT (48), TOMKINS et al. (198) and SHARMA et al. (50) had solubilized and purified this enzyme. It can be obtained

in a soluble form by ultrasonic treatment (RAMAN et al. (49)).

11β-hydroxylation occurs throughout the cortex (199-202). It has been speculated that two 11β-hydroxylases exist in the Z. G., one specific for progesterone, the other, throughout the cortex, is specific for DOC (GRANT (17)). SWEAT et al. (203) reported the separation from a soluble extract of acetone-dried adrenal mitochondria two fractions, designated F-40, and F-80, which were precipitated with 40% and 80% ammonium sulfate respectively. They observed that these two fractions were highly active and require Mg^{2+} and NADPH for their activities. The multiple-component nature of 11β-hydroxylase had been re-examined by TOMKINS et al. (198, 204), who found that the combination of three protein fractions and an unknown heat-stable factor were essential for 11β-hydroxylase activity in the presence of O_2 and NADPH. An NADPH-specific flavoprotein (F_p) and a non-heme iron-protein (NHIP) have been shown to be constituents of the 11β-hydroxylase system (OMURA et al. (134), SUZUKI et al. (205), SWEAT et al. (206)). These two components with the cytochrome P_{450} made up the electron transport system for 11β-hydroxylation.

The effect of ascorbic acid on 11β-hydroxylase has not been settled (207 - 211). DOC has a strong inhibitory effect on the 11β-hydroxylation (HAYANO et al. (30), PERON et al.

(212)). Moreover, SHARMA et al. (213) had shown that several C_{19} steroids, e.g., testosterone, are competitive inhibitors of the 11β-hydroxylation of DOC.

(b) 21-hydroxylase (EC 1.99.1.11). This enzyme, which is present in all eutheria (5, 151) and throughout the cortex (GIROUD et al. (185, 186)), was found to be associated with the 15,000 g supernatant (RYAN et al. (183)) of beef adrenals. HAYANO et al. (100) believed that it may be adsorbed on mitochondrial surface. Attempts to obtain the activity of the microsomal particles in solution have been unsuccessful (184).

The 21-hydroxylation activity of progesterone is optimal at pH 6.9 to 7.4 (187) and inhibited by carbon monoxide (RYAN et al. (183)). It requires atmospheric oxygen and NADPH (183) but is inhibited by a high concentration of NADPH (188). It is stimulated by a protein factor (154). Its partial inactivation or inhibition in a dilute homogenate, or during incubation at 37°C., had been observed by TSANG (189). The requirement of P_{450} for its activity is well established, but the role of the P_{450} reductase system on this enzyme requires further clarification (BRYSON et al. (190)).

(c) 18-hydroxylase. PSYCHOYOS et al. (214) and others (215, 216, 49, 102) had shown that 18-hydroxylase and 18-oldehydrogenase (RAMAN et al. (49)) are associated mainly with

the mitochondrial fraction (4,000 g; 10 min.) of rat adrenal. This 18-hydroxylase was reported by WILSON et al. (216) to be located in the "heavy" mitochondria of the rat adrenal homogenate, but NAKAMURA et al. (215) found the presence of the enzyme in the nuclei and microsomes as well.

The 18-hydroxylation occurs in the rat adrenal sections and homogenates (108, 109). Since 18-hydroxycorticosterone (109, 110) is produced in the Z. G. and the 18-hydroxy,11-deoxycorticosterone in the Z. F. (108 - 110), it is evident that the 18-hydroxylase is present throughout the rat adrenal cortex.

RAMAN et al. (49) noticed that there was no significant loss in 18-hydroxylase and 18-ol-dehydrogenase activities in mitochondria following ultrasonic treatment, and these activities were located principally in the sediment after centrifugation at 105,000 g for 60 minutes. This would indicate that intact mitochondria were not required for 18-hydroxylase and 18-ol-dehydrogenase and they are possibly closely bound to either mitochondrial membranes or to the cristae.

The 18-hydroxylation requires the same NADPH-cytochrome P_{450} reducing system as 11β-hydroxylation (184). Such a requirement for the eventual synthesis of aldosterone from corticosterone in cell-free systems have been demonstrated by

several groups (RAMAN et al. (49), PSYCHOYOS et al. (180), TALLAN et al. (181) and GREENGARD et al. (182)). The 18-hydroxylase activity of rat at saturating substrate concentration is about as high as the 11β-hydroxylase activity (184). RAMAN et al. (49) observed that SU 8000 [(3-16-chloro-3-methyl-2-indenyl)pyridine] inhibited the 18-ol-dehydrogenase and, to a lesser extent, the 18-hydroxylase. The ions of some transition elements (e.g., Cu^{2+}), as much as 1 x 10^{-3} M, would inhibit the conversion of corticosterone to 18-hydroxycorticosterone and aldosterone (49).

D. Adrenal cortex steroid hydroxylation characteristics

(a) Requirements: Most of the steroid hydroxylases have been shown to have common requirements and the same mechanism in their activities (184).

(i) Oxygen. SAFFRAN and BAYLISS (28) demonstrated that rat adrenals failed to produce corticoids when air or oxygen environment is replaced by nitrogen. Using H_2O^{18}, O_2^{18} and D_2O, HAYANO et al. (217) had proven that molecular oxygen, but not H_2O, is utilized for the 11β-hydroxylation of steroids. This was confirmed by SWEAT et al. (218). HAYANO et al. (219) reported that 21-hydroxylation also required molecular oxygen.

(ii) NADPH. NADP or NADPH are necessary for the 21-, 11β- and 18-hydroxylations of steroids (220 - 223). SAFFRAN

and BAYLISS observed no corticosteroidogenesis when rat adrenal homogenates were incubated alone (28).

For the source and distribution of NADPH, PERON and MCCARTHY (224) stated that any substrate permeable to the adrenal cortical mitochondrial membrane and which can be oxidized by the proper dehydrogenase in a NADP-linked reaction will give rise to intramitochondrial NADPH. The intramitochondrial NADP appears to be very firmly associated with mitochondrial enzyme (225). The idea that average cellular concentration of NADPH in rat adrenal gland is directly related to the rate of synthesis of adrenal steroids (226) was not confirmed by HARDING and NELSON (227, 228). Using the method of PURVIS (229), PERON et al. (226) showed that the total pyridine nucleotides appear to be approximately equal in distribution in mitochondrial and supernatant fraction. Essentially the same pattern of pyridine nucleotide content was obtained in cell fractions from normal and hypophysectomized animals (PERON et al. (230)).

With regard to the role of NADPH, PERON et al. (231) found that adrenal tissues maximally stimulated with ACTH will be further stimulated by $NADP^+$ + G-6-P. Conversely, adrenal tissues maximally stimulated with $NADP^+$ + G-6-P will still respond to ACTH (230). These authors also found that when the swelling of mitochondria is maximal, at a

concentration of Ca^{2+}(11 mM), exogenous NADPH would penetrate into mitochondria and be utilized by the hydroxylase system (232). According to GUERRA et al. (233), the exogenous NADPH would bring about hydroxylation by other mechanism when mitochondria were maximally swollen. NADPH participates in the mechanism of the transfer of electron (234). According to SIH et al. (235), the dual role of NADPH is: (a) to serve as an accessory capacity, keeping P_{450} in the Fe^{2+} level of oxidation, and (b) NADPH directly involved in steroid hydroxylation reaction to generate the highly reactive hydroperoxo complex, $P_{450}Fe^{2+}$-O-OH.

The two known pathways for the generation of NADPH through the action of the dehydrogenases are: the direct oxidative pathway, and the Krebs tricarboxylic acid cycle. The source of the hydrogen atoms must be from the substrates of the specific dehydrogenases, the substrate being derived from glucose.

As stated in HAYNES' hypothesis (236), the stimulatory effect of ACTH on corticosteroid biogenesis in the adrenals is, essentially, to increase the breakdown of glycogen to G-6-P, which, upon oxidation, produces more NADPH. The adrenal cortex contains a high level of glucose-6-phosphate dehydrogenase (G-6-P-D), and 6-phosphogluconic dehydrogenase (237, 238), which show highest activities in zona fasciculata-reticularis (239, 240). From their recent studies with bovine

and rat adrenals, MCKERNS (241) found that G-6-P-D has the highest activity, whilst 6-phosphogluconic dehydrogenase, isocitric dehydrogenase, and malic dehydrogenase are in the decreasing order of activity.

It has been shown that Krebs tricarboxylic acid (TCA) cycle intermediates support mitochondrial 11β-hydroxylation of DOC (192, 194, 196, 244, 245) and that the reactions may be affected by uncouplers of oxidative phosphorylation (BROWNIE et al. (195)). The role of the TCA cycle intermediates would be to generate the intramitochondrial NADPH required for the steroid hydroxylations (225, 245, 246). Fumarate is required in relatively high concentrations for 11β-hydroxylations by mitochondrial (194) preparations, but not in pure acetone powder extracts. It has been assumed to play no direct role in the 11β-hydroxylation (247) other than related to the production of NADPH (48), but HAYANO et al. (219) suggested that fumarate serves in the protection of the O_2-bound enzyme rather than as a reductant for NADP. The order of relative efficiency of the TCA intermediates, according to SWEAT et al. (194), is: fumarate, malate, succinate, isocitrate, cisaconitate and citrate (30, 248). In aged tissue preparations, malate was somewhat less active still (HAYANO et al. (249)).

Both energy-linked transhydrogenase reaction (OLDHAM et al. (242)) and non-energy-dependent reactions are possible

mechanisms for the NADPH formation in adrenal cortex mitochondria. SAUER et al. (243) suggested that the substrate utilized determines the method of NADPH formation in vitro. It was also noted that the selection between the pathways of NADPH generation for 11β-hydroxylation is probably species dependent (CHENG (133)).

(iii) Calcium ions (Ca^{2+}). PERON et al. (232) observed that Ca^{2+} have an effect on utilization of both Krebs TCA cycle intermediates and NADPH and concluded that Ca^{2+} are required for corticosteroid biosynthesis in vitro (250). The locus of Ca^{2+} action was at the mitochondrial level. However, the possible influence of Ca^{2+} on 11β-hydroxylation which is separate from its swelling effect on mitochondria has been speculated (251).

(b) Mechanism of adrenal cortex steroid hydroxylation. Various investigators have studied the mechanism of steroid hydroxylations (252, 192, 253, 31, 254, 255, 48).

The reaction apparently proceeds by electrophilic attack whereby the incoming hydroxyl group replaces the hydrogen at the position to be hydroxylated with retention of configuration (256). According to MASON (257), steroid hydroxylation (mixed-function oxidations) reactions have the following stoichiometry:

$$AH + NADPH + H^+ + O_2 \longrightarrow A\text{-}OH + NADP^+ + H_2O$$

where A stands for a primary, secondary, or tertiary carbon of the steroid molecule. The validity of the stoichiometry for this equation was established by COOPER et al. (258), who showed that the ratio of NADPH oxidized to 17,21-dihydroxy-progesterone formed to O_2 reduced is indeed 1:1:1 as predicted by the above equation.

Incorporation of one atom of $^{18}O_2$ into the steroid molecule has been demonstrated for adrenal 11β-hydroxylations (218, 259). For adrenal 21-hydroxylations (258) and 11β-hydroxylations (260) it has been shown that one mole of NADPH and one mole of O_2 are utilized for each mole of hydroxylation product formed in accordance to the above equation. Although the fate of the second oxygen atom cannot be directly determined, it is most likely that it is reduced to water by NADPH (184).

(i) Electron transport system of mixed-function oxidase. Stemmed from the works of RYAN and ENGEL (183), GARFINKLE (261), KLINGENBERG (262), ESTABROOK and co-workers (263), OMURA and SATO (264) isolated a cytochrome-like substance from liver microsomes and called it P_{450} because of its strong absorption at 450 mμ in the CO-complex form. Later, OMURA et al. (134) and HARDING et al. (265) isolated a similar substance in beef

and rat mitochondria necessary for the 11β-hydroxylation of DOC. More recently, OMURA and collaborators (134) and NAKAMURA and OTSUKA (266) had isolated from beef, rat and porcine mitochondria three protein fractions: a non-heme iron-protein (NHIP), called adrenodoxin (205), a NADPH-specific flavoprotein dehydrogenase (F_p) and a cytochrome-protein CO-complex called P_{450}. These three fractions, upon recombination, support the 11β-hydroxylation of DOC to corticosterone (134, 267). From such studies, they have evolved the scheme of electron flow originating as a result of the oxidation of intramitochondrial NADPH and the passage of electrons from one component to the next through the terminal cytochrome P_{450}. According to SIMPSON et al. (268), P_{450} is: (1) responsible (269) for oxygen activation in steroid mixed-function oxidases, of both mitochondrial and microsomal level, (2) the site of substrate binding in these enzyme systems. P_{450}, the enzyme for the mixed-function oxidases, acts, in conjunction with molecular oxygen and other enzymes, in directing the stereospecific transfer of hydroxyl ions at the 11β- and other positions of the steroid molecule (270). The reactions involved could be represented by the following scheme (271) in Figure 3, where arrows indicate the direction of electron flow. The central role of cytochrome P_{450} in the hydroxylation of these substrates,

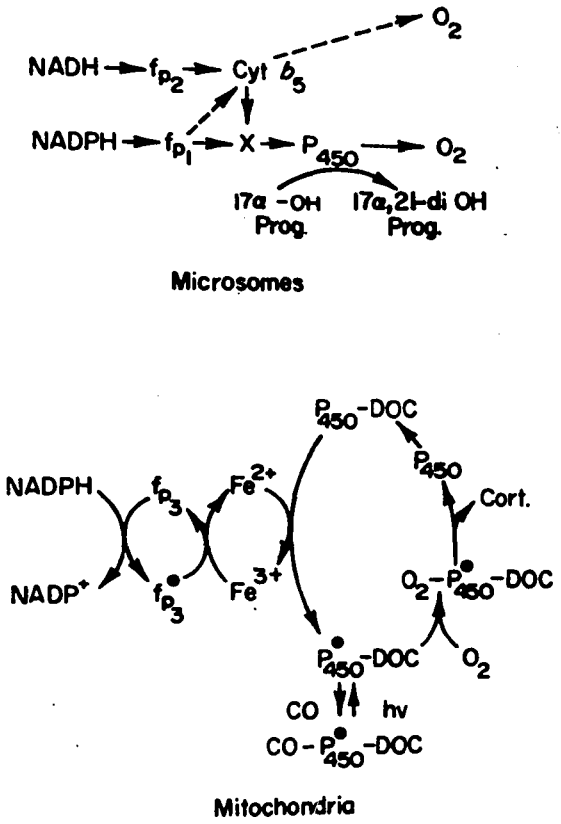


Fig. 3. Scheme of electron transport in microsomal and mitochondrial mixed-function oxidases of bovine adrenal cortex(271).

with particular emphasis on the cellular distribution of this pigment, has pointed to the effectiveness of compartmentalization of these reactions. However, the intimate details of how cytochrome P_{450} activates oxygen and interacts with steroid substrates remain unresolved (272).

(ii) Supply of reducing equivalent in mitochondria. In contrast to other types of mitochondria from mammalian tissues, mitochondria from adrenal cortex possess two distinct types of electron transport (respiratory) chains: one type similar to the respiratory chain observed in liver, heart, kidney or muscle mitochondria, the second type of electron transport chain is functional (134, 169) in transfer of reducing equivalent from NADPH to cytochrome P_{450} for the 11β- and 18-hydroxylation of DOC.

As first suggested by HARDING et al. (134), the energy generated during a Krebs TCA cycle intermediate oxidation by the conventional type of respiratory chain may be utilized by the energy-linked pyridine nucleotide transhydrogenase to aid in maintenance of a high level of NADPH for the operation of the hydroxylation pathway. Recently, SIMPSON et al. (272) have proposed the scheme of metabolic pathways in bovine adrenal cortex shown in Figure 4. In this scheme, it can be seen that the only link between the respiratory chain and

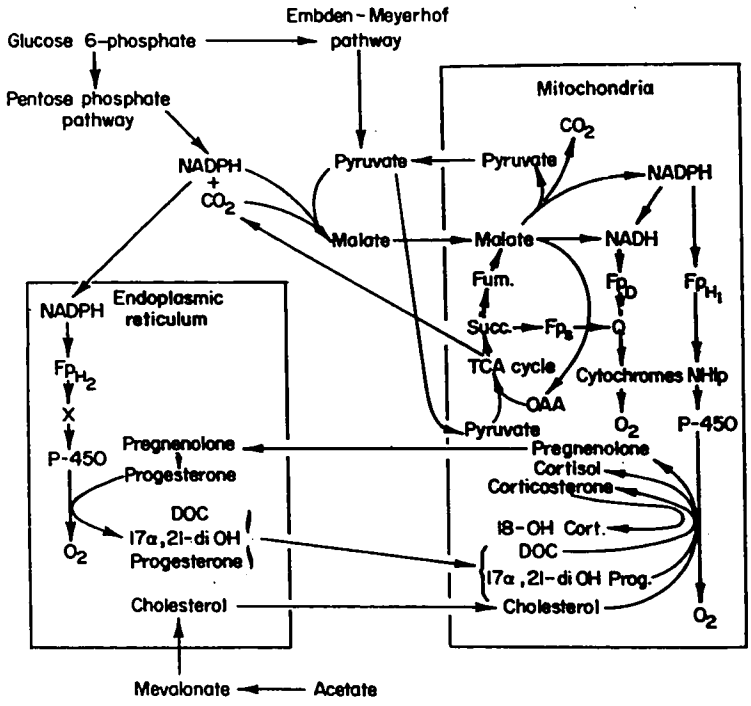


Fig. 4. Scheme showing proposed metabolic pathways in bovine adrenal cortex(272).

the hydroxylation pathway (i.e., the cytochrome P_{450} chain) is through the transhydrogenation between NAD and NADP, i.e., connected by energy-linked pyridine nucleotide transhydrogenase (ELTH) (SWEAT et al. (194), OLDHAM et al. (242), and PURVIS et al. (273)). This seems to be supported by the high activity of pyridine transhydrogenase detected by HARDING et al. (228) in rat adrenal mitochondria. The transhydrogenase activity provides a mechanism which permits the hydroxylating enzymes to compete favourably with the respiratory enzymes for reducing equivalents (274). Malate is oxidized to pyruvate in the bovine and rat adrenal gland mitochondria with the concurrent formation of intramitochondrial NADPH necessary for steroid 11β-hydroxylation. PERON et al. (275) described a possible "malate shuttle" mechanism by which the cytoplasmic malic enzyme could act in vivo to produce malate from pyruvate, bicarbonate and extramitochondrial NADPH. This malate would enter the mitochondria to generate the intramitochondrial NADPH.

E. Conclusions

From the information based on the study of cell fractions and the previously presented views of adrenal corticosteroidogenesis, the scheme shown in Figure 5, partially adapted from LONG and JONES, 1969 (12), for the biochemical pathways in the synthesis of adrenal steroids can be drawn (FAWCETT et al. (12)).

The several steps in the biosynthesis of cholesterol from acetate involve enzymes that are either in the membranes of the smooth reticulum (microsomes) or in the surrounding cytoplasmic matrix (supernatant). The cholesterol formed probably moves to mitochondria for cleavage of the side-chain in a series of hydroxylation reactions. The resulting pregnenolone is acted upon by enzymes which also reside in the reticulum or in the matrix, leading to the formation of progesterone. Continuing along the path to adrenal steroids involves the action of successive hydroxylases of which the first (21-hydroxylase) is the reticulum (RYAN et al. (183)) and the others are mitochondrial (12).

However, much still remains to be learnt about how substrates move from one organelle to another, for the successive steps in steroid synthesis, and how this traffic is controlled.

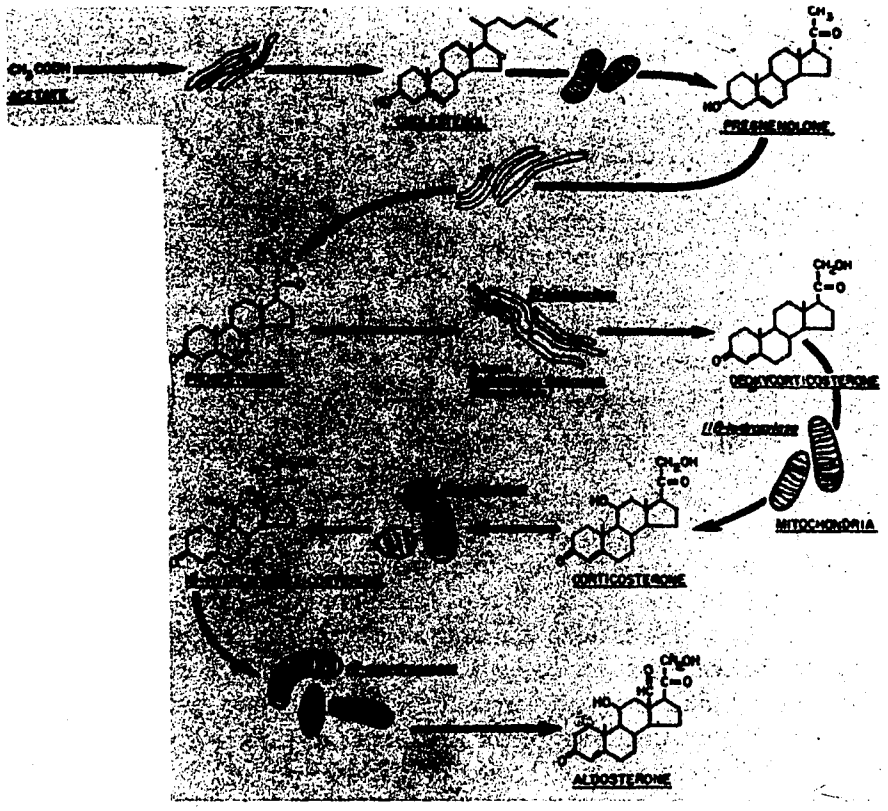


Fig. 5. Schematic of the biochemical pathways in the synthesis of adrenal steroids. Mitochondria or smooth endoplasmic reticulum are depicted to indicate the probable sites of the enzymes involved in the various steps (12).

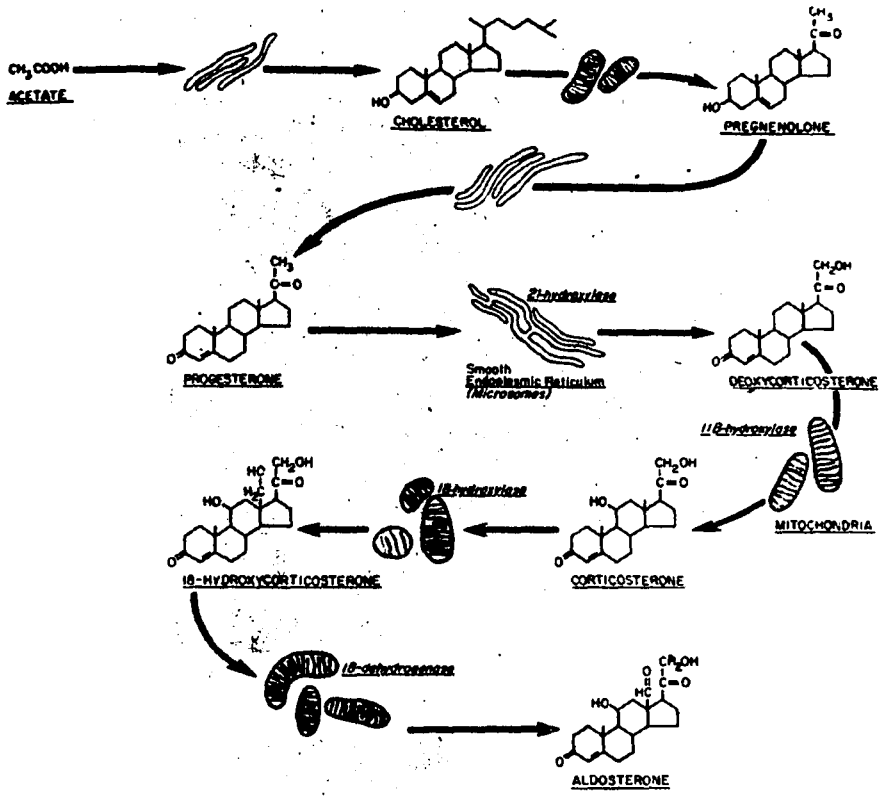


Fig. 5. Schematic of the biochemical pathways in the synthesis of adrenal steroids. Mitochondria or smooth endoplasmic reticulum are depicted to indicate the probable sites of the enzymes involved in the various steps (12).

CHAPTER III

MATERIALS AND REAGENTS

1. Buffer solution

(a) Krebs-Ringer bicarbonate glucose (KRBG) solution of pH 7.4 (consisting of 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 24.8 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 11.1 mM glucose in distilled water) was the buffer medium used for all the incubations in this Thesis. The buffer was freshly prepared from the mother solutions (38), and gased with 95% O_2 - 5% CO_2 before use.

(b) Phosphate buffer of pH 7.6 (consisting of 10.1 mM KH_2PO_4, 56.9 mM Na_2HPO_4) was used as the medium for the enzymatic reduction of the 20-ketone group of progesterone or 11β-hydroxyprogesterone.

2. Cofactors and enzymes

The following were purchased from Sigma Chemical Company:

NADP (nicotinamide adenine dinucleotide phosphate monosodium salt),

β-NADH (beta-nicotinamide adenine dinucleotide, reduced form),

G-6-P (glucose-6-phosphate disodium salt),

G-6-P-D (glucose-6-phosphate dehydrogenase, type V),

20β-hydroxysteroid dehydrogenase, type II.

3. Non-radioactive Δ^4-3-ketosteroids

These were obtained from Steroids Inc. or Merck in crystalline forms. They were used mostly as internal standards and sometimes for the dilution of the respective radioactive steroids used as precursors in incubations.

Known amounts of steroids were weighed in a Micro Gram-atic Balance (made by E. Mettler, Zurich), dissolved in distilled ethanol and stored in stoppered flasks at 4°C. The purity of a steroid was checked by testing 60 µg. of the compound in an appropriate thin-layer chromatography (TLC) system. When no ultraviolet light (250 mµ) absorbing impurities were visualized, the compound was used without further purification.

The TLC systems (Table I) for this purpose were: TLC - 1 for aldosterone, corticosterone, 11β-hydroxy-progesterone and 11-deoxycorticosterone; TLC - 6 for progesterone; TLC - 2 for aldosterone-18,21-diacetate, corticosterone-21-monoacetate and 11-deoxycorticosterone-21-monoacetate.

4. Radioactive steroids (New England Nuclear Corp.)

Whenever possible, ^{14}C-labeled steroids were used as precursors in incubations, and ^{3}H-labeled steroids as tracers

for the correction of losses incurred during the purification processes.

The labeled steroids (only free forms were used) were purified mainly by TLC described above and paper chromatography (PC) (e.g., toluene propylene glycol-methanol system for aldosterone), aided by radioautography and radiochromatogram scanning for the recognition of purity.

The specific activities of the labeled steroids obtained from the suppliers are as follows:

1,2-^{3}H-steroids	10 curies/mM (11-deoxycorticosterone), 20 - 30 curies/mM (aldosterone, corticosterone), 25 - 40 curies/mM (progesterone),
4-^{14}C-steroids	20 - 30 mc/mM (11-deoxycorticosterone), 30 - 50 mc/mM (aldosterone, corticosterone, progesterone).

Radioactive precursors when diluted was with known amounts of the non-radioactive steroids to a suitable specific activity. The final specific activities were determined before use.

5. Radioactive acetic anhydride (New England Nuclear Corp.)

Acetic-^{3}H anhydride, specific activity 50 mc/mole, was used undiluted (in 12% benzene solution). Acetic-^{14}C anhydride, specific activity 10 mc/mole, was usually diluted about 10 times with non-radioactive acetic anhydride. Anhydrous benzene was then added to give a final concentration of 10% acetic anhydride in dry benzene (v/v).

Radioactive acetic anhydride vial was securely stored in brown, tightly waterproof bottles containing desiccant at -20°C.

6. Reagents for extraction, chromatography and chemical reactions

Unless otherwise stated, the following solvents (reagent grade) were redistilled in an all-glass apparatus fitted with a fractionation column of Vigreux type. Certain solvents which required special treatment are described below.

Ethyl acetate was washed once with 1/10 volume of distilled water to neutrality, dehydrated with anhydrous sodium sulfate, filtered, redistilled and collected over anhydrous sodium sulfate on filter paper in a funnel.

Toluene for chromatography was washed once with 1/10 volume of reagent grade H_2SO_4, once with 1/10 volume of 10%

Na_2CO_3 and finally with 1/10 volume of distilled water to neutrality. It was then dehydrated with anhydrous sodium sulfate, filtered and redistilled.

Acetic anhydride (non-radioactive), benzene, pyridine and acetic acid were distilled according to KLIMAN and PETERSON (78), and stored at 5°C. in a vacuum desiccator over a layer of calcium chloride.

7. Scintillation fluid for counting

Four grams of 2,5-diphenyloxazole and 0.1 gm. of 1,4-bis-2(5-phenyloxazole)benzene (Packard Instrument Co., La Grange, Ill., U. S. A.) were dissolved in 1 litre of redistilled toluene.

8. PORTER-SILBER reagent

This consists of the following four parts:

(a) Diluted sulfuric acid - 62% (19 ml. redistilled H_2O:31 ml. concentrated H_2SO_4),

(b) Phenylhydrazine HCl, recrystallized from ethanol 4 times,

(c) Phenylhydrazine-sulfuric acid solution (16 mg. of reagent (b) plus 10 ml. of reagent (a)) prepared each day immediately before use,

(d) Ethanol, redistilled 3 times.

9. Iso-nicotinic acid hydrazide reagent

This consists of the following four parts:

(a) Absolute aldehyde free, 3 times distilled methanol,

(b) Isonicotinic acid hydrazide reagent (Taylor Chemical Co.),

(c) Acid alcohol (0.625 ml. conc. HCl in 1 litre of absolute ethanol).

500 mg. of isonicotinic acid hydrazide is dissolved in 1 litre of acid ethanol. The reagent appears to be stable for a few weeks when kept in frigidaire.

10. Periodic acid reagent

2.28 gm. of periodic acid is dissolved in 98 ml. distilled water and 2 ml. distilled pyridine followed by thorough shaking. The container is well stoppered, wrapped in tin foil and kept in the cold room.

11. Chromium trioxide oxidation reagent

This reagent is made up of 0.5% chromium trioxide in 95% acetic acid.

To a 10 ml. volumetric flask fitted with a glass stopper, 0.5 ml. distilled water was added. 100% re-distilled acetic acid (78) was added, in small portions with stirring, to 0.5 gm. CrO_3 and transferred by a Pasteur pipette to the volumetric flask, until all the oxide has been dissolved and quantitatively transferred to the flask. The final volume of 10 ml. is made up with the acetic acid,

including the rinsing of the pipette. The flask is wrapped in tin foil and well stoppered after thorough shaking. It is kept in a frigidaire. The reagent prepared in this way can be stored for a few months.

12. Glassware and vials

All glassware was thoroughly cleaned by the following procedure. Glassware after use(or new ones) was rinsed with water, and then cleaned with the detergent and water before being put into the chromic acid (concentrated) cleaning solution for 12 hours. Finally, they were rinsed thoroughly with tap water, and six times with distilled water before the final drying in the oven.

Quickfit Corning glass corked tubes model MF 24/0/4 with a conical tip, capacity 7 ml., were used for micro-acetylation of steroids when minute amounts of labeled acetic anhydride (0.03 ml.) were used. Separatory funnels (60 ml. capacity) fitted with Teflon tap (Fisher No. 10-437-10) were used for the extraction of steroids. Vacuum extractors (Scieitific Glass Blowing, Montreal) of about 6 ml. capacity and having sintered glass filters were used for elution from thin-layer chromatoplates.

CHAPTER IV

METHODS

(A) General

For the separation, purification and identification of the steroids, thin-layer and paper chromatographies were mostly used, either alone or in conjunction with different treatments which modify the steroid molecules chemically.

1. CHROMATOGRAPHY

1. Chromatography Systems (Tables I - III and Figure 7)

Both thin-layer and paper chromatographic systems have been used for the separation and purification and identification of steroids in this Thesis, although thin-layer chromatography was most often used.

(a) Thin-layer chromatography (TLC).

(i) Procedure and systems used. Glass plates (20 x 20 cm.) were coated with Merck silica gel G, 0.5 mm. thick, containing 1% of lamp phosphor (Sylvania Electric Products Inc.) to facilitate the detection of the α,β-unsaturated steroids under ultraviolet (U.V.) light (250 mu Mineralight UVS. 11 San Gabriel, Calif., U.S.A.). The plates were dried overnight and activated by heating for 30 minutes under an infrared lamp before use. The application line was

2 cm. above the edge of the glass plates. The TLC systems require no equilibration time, but in order to have reproducible running rates of the different steroids, it was found to be necessary to line the walls of the chromatography tank with chromatography paper (Whatman No. 2, 23 x 24 x 12 cm.) saturated with the solvent system. Plates were developed at room temperature (21°C.) by ascending chromatography until the solvent front was 17 cm. from the origin. The time of development varied from 45 to 90 minutes, depending on the system used. A list of the TLC systems used is given in Table I. The different systems will be referred to by the Arabic numbers assigned to each system.

(ii) Detection of steroids on thin-layer chromatograms: (1) Ultraviolet light absorption. The radioactive α,β-unsaturated steroids isolated on thin-layer chromatograms were visualized in the dark as U.V. absorbing spots (Mineralight UVS.11). The localization was facilitated by the addition of 10 - 20 µg. of rhe respective non-radioactive internal pilots whenever possible or necessary.
(2) Radioautography. The radioactive (C^{14}) products on thin-layer plates were located with x-ray films (Cronex 2DC medical x-ray film, DU Point). The plate was marked with a C^{14} marker (e.g., highly radioactive C^{14}-progesterone) to ascertain the actual position of the film on it. The plate

was placed in a light-tight x-ray cassette (Halsey Rigidform Cassette, 8 x 10", Brooklyn, N.Y., U.S.A.) for a length of time depending on the quantity of radioactivity applied, but not less than 24 hours. The film was then developed by standard x-ray film developing techniques in an automatic processing machine (Kodak) in the X-Ray Department, Montreal Children's Hospital. The film was not sensitive enough to detect the ^{3}H-labeled compounds used in this Thesis.

(iii) Elution from thin-layer chromatogram. Silica gel on areas containing the steroids were extracted from the plate with a vacuum extractor (Scientific Glass Blowing, Montreal) having a sintered glass filter. The steroids were eluted from the silica gel with 3 x 2 ml. ethyl acetate-ethanol (3:2, v/v). The eluate was evaporated under a stream of <u>dry</u> air at 40°C.. In most instances the losses could be corrected by the addition of a radioactive steroid tracer prior to the processing of the extract.

(b) Paper Chromatography (PC)

(i) Procedure and systems used. The type of Whatman filter paper used depended on the system to be used. For systems requiring impregnation, e.g., the toluene propylene glycol (T.P.G.) system, Whatman No. 1 was used; for systems not requiring impregnation,e.g., Bush A system, Whatman No. 2 paper was used. The paper was used unwashed after prior

scanning under ultraviolet light (250 mμ) to check if it was free of U.V. absorbing or fluorescent impurities. The paper was cut into strips 45 cm. long, and the width was dependent on the number of spots to be applied on the paper. However, generally 2 cm. width was allowed for each spot cut out to prevent mixing of the spots when the solvent front descended. The common head was 12 cm. long and the line of application was 1 cm. from the common head. A list of paper chromatographic systems used is given in Table II.

(ii) Detection of steroids on paper chromatograms:
(1) Ultraviolet light absorption. The radioactive α,β - unsaturated steroids isolated on paper chromatograms were visualized on a U.V. filter (18" x 6½",(ICS) D. 631-512 9863 OI). The localization was facilitated by the addition of 10 - 20 μg. of the respective non-radioactive internal pilots whenever possible or necessary.
(2) Radiochromatogram scanning. Radioactivity on paper strips was scanned with a radiochromatogram scanner (Packard Model 7200). The sensitivity was adjusted according to the expected amount of radioactivity in the compounds studied. The speed was usually set at 0.5 to 1 cm./min.

(iii) Elution from paper chromatogram. Areas on the strips containing the steroids were cut out, folded in zig-zag form with forceps and placed in large tubes that can be

stoppered tightly. Sufficient ethyl acetate-ethanol (3:2,v/v) was added to immerse the paper cuttings completely for 30 minutes with the tubes stoppered. The tubes were then shaken vigorously for about 2 minutes. The paper was taken up by clean forceps to the mouth of the tubes and allowed to stand for about 10 minutes during which the rinsings of the stoppers and the forceps would have drained into the tubes. Drops of the ethyl acetate-ethanol (3:2,v/v) solution were added by means of Pasteur pipette along the top edge of the zig-zag paper until 10 ml. of the solution had been added. The paper was allowed to drain off all the solution added to it. After removing the paper (which could then be discarded) and rinsing the mouth of the tubes with a few drops of the ethyl acetate-ethanol (3:2, v/v), the tubes were dried under a stream of dry air at 40°C..

2. Chemical Modifications

(a) Acetylation of steroid compounds

(i) With radioactive acetic anhydride. The procedure described by STACHENKO et al. (95) was followed. Steroids were concentrated at the tip of an acetylation tube, which was allowed to get thoroughly dry at least 4 hours in a vacuum desiccator. The pipettes, flamed for about 30 seconds immediately before use, were fitted in an "ultra-micro pipet-filler". 0.025 ml. of distilled pyridine and 0.03 ml. of acetic-^{3}H anhydride were added to the tube, which was

stoppered immediately, and gently shaken to allow complete mixing of materials at the tip. The mixture was incubated overnight at 50°C. in a constant temperature Dri-thermolyne bath (Dubuque, U.S.A.) in the dark.

The extraction was carried out according to the method of KLIMAN and PETERSON (78).

(ii) With non-radioactive acetic anhydride ("macro"-acetylation)(76). 0.30 ml. of distilled pyridine and 0.15 ml. of acetic anhydride were used. After standing overnight at room temperature in the dark, the acetylation was stopped with 1 ml. of distilled ethanol and the excess reagent was dried under a stream of air. Should the dried tube still exhibited the pyridine odor, another 1 ml. of ethanol was added and the tube dried as before. The acetylated extract was applied to either paper or thin-layer chromatography directly from the acetylation tube.

(b) Oxidation of the 11β-hydroxyl group of the steroid molecule

Oxidation of the 11β-hydroxyl group of the steroid 21-acetates or the 21-methyl steroid to the 11-keto group was carried out according to the method described by KLIMAN and PETERSON (78).

(c) Reduction of progesterone and 11β-hydroxyprogesterone

with 20β-hydroxysteroid dehydrogenase (Sigma)

Because of the lack of hydroxyl groups which may be readily acetylated with acetic anhydride on the progesterone or 11β-hydroxyprogesterone molecule, the two steroids were first reduced enzymatically to the 20β-hydroxyl derivatives before acetylation.

The eluates of the spots corresponding to progesterone or 11β-hydroxyprogesterone were incubated in a test tube with 0.1 mg. of 20β-hydroxysteroid dehydrogenase and 0.2 mg. (0.256 µmoles) of β-NADH (excess if necessary) in 1 ml. phosphate buffer pH 7.4 (Chapter III, 1) overnight at room temperature, keeping the tubes tightly stoppered and in the dark.

The reduction product was quantitatively transferred to a separatory funnel (60 ml. capacity) with 1.5 ml. distilled water and extracted with 2 x 6 ml. dichloromethane. The dichloromethane extract was concentrated at the tip of a 15 ml. centrifuge tube to facilitate chromatographic application.

(d) Periodic acid oxidation

Etiolactones of 18-hydroxylated steroids were formed by the method described by TAIT et al. (34).

Equal volumes of 0.5 ml. distilled methanol and then 0.5 ml. of periodic acid (HIO_4) were added to the 18-hydroxylated steroid samples. After well mixing, they were placed

in the dark for 18 hours. At the end of the oxidation period, 1 ml. of distilled water was added to each tube before proceeding to the extraction of the oxidation mixture with dichloromethane. The method of extraction is identical to that described for chromium trioxide (see (b) above). The standard etiolactone of aldosterone (300 ug.) were obtained by using 1 ml. of methanol and 1 ml. of periodic acid for the oxidation.

After the extraction of the oxidized samples, TLC - 2 was applied. In this system, the etiolactone of aldosterone had an R_f = 0.26, etiolactone of 18-hydroxycorticosterone gave an R_f = 0.09, and etiolactone of 18-hydroxy,11-deoxy-corticosterone had an R_f = 0.35.

3. Quantification of the Steroids

(a) Ultraviolet light determination

Steroids with α, β - unsaturated grouping maximally absorb U.V. light at 238 - 240 mμ. The method of U.V. determination of the steroids follows that described by ELLIOT et al. (64).

Silica cuvettes and Unicam Spectrophotometer Model SP 500 with hydrogen lamp setting were used. The samples were read in cuvettes containing 1 ml. methanol (3 times distilled) at 225, 240 and 255 mμ, against paper or thin-layer

silica gel blank. Two sets of standards, say of progesterone, of 2, 5 and 10 ug. were read similarly, one set before and one set after the reading of the actual samples, to check the agreement of the precision of the machine.

After applying the ALLEN Correction Factor (276) to the readings, the actual quantities of the steroids in the samples were read off from the standard curve.

(b) Iso-nicotinic acid hydrazide (INH) reaction (65, 65a)

The reaction quantitatively determines the Δ^4-3-ketosteroids on the basis of the rapid formation of their hydrazones from INH in acidified alcohol. A yellow colour having an absorption maximum at 380 mμ.

Two sets of standards, structurally most similar to the steroid studied, of 2, 5 and 10 ug. were prepared and dried in tubes fitted with glass stoppers. To these tubes, the dry samples of steroids and the blank eluates were added 0.8 ml. of INH reagent (Chapter III, 9) and the tubes were shaken. After 1 hour of standing in stoppered tubes, the samples were read at 350, 380 and 410 mμ. in a Unicam Spectrophotometer Model SP 500. The readings of the standards were against the reagent blank while the samples were read against the blank eluate.

After applying the ALLEN Correction Factor (276) to

the readings, the actual quantities of the Δ^4-3-ketosteroids in the samples were determined from the standard curve of cortisol.

(c) PORTER-SILBER reaction

The PORTER-SILBER reaction (75) was followed to detect and measure the dihydroxyacetone side-chain of steroids (cortisol, as in the determination of the specific activity of a batch of labeled acetic anhydride). The reaction is also given by 18-hydroxy,11-deoxycorticosterone.

To the dry steroid residue and the dry eluate of paper or thin-layer chromatography blank was added 0.3 ml. ethanol. They were well mixed. Then 0.45 ml. of phenylhydrazine-sulfuric acid solution was added to each tube followed by a thorough mixing. The tubes, tightly stoppered, were kept at room temperature overnight in the dark. The procedures were also applied to the two sets of cortisol standards of 2, 4 and 8 ug.

On the following day, the reaction mixtures were read in a Unicam Spectrophotometer Model SP 500 at 370, 410 and 450 mμ set with the tungsten lamp against the reagent and eluate blanks. Quartz cuvettes were used. If the reaction mixtures gave too high readings, they could be diluted with the reagent blank (prepared in excess) and proceed with the readings.

After applying the ALLEN Correction Factor (276) to the readings, the actual quantities of the steroids in the samples were read against the cortisol standard curve.

(d) Determination of specific activity of ^{3}H- or ^{14}C-labeled acetic anhydride

The determination of the specific activity of the commercial ^{3}H- or ^{14}C-labeled acetic anhydride used in these experiments have been described by STACHENKO et al. (95). Generally, the specific activity of ^{3}H-acetic anhydride used was 50 uc/µmole and that of the ^{14}C-acetic anhydride was 10 µc/µmole.

(e) Estimation of radioactivity in samples

Aliquots of the steroid eluate after chromatography were transferred into 20 ml. glass vials (Packard, Cat. No. 6001015), dried under a stream of air under infrared light at about 50°C.. Fifteen millilitres of scintillation mixture (Chapter III, 7) was introduced into the vials. Since most of the samples contained ^{3}H and ^{14}C, simultaneous counting (78) of ^{3}H and ^{14}C was done in a Packard Tricarb Scintillation Spectrophotometer, Model 4322, at a single voltage (1580 volts), using the discriminator ratio method of OKITA (278). Each sample was counted for sufficient time (20 minutes) so that a standard error of the mean of less than 2% was achieved in both channels. Efficiencies

for ^{14}C and ^{3}H under dual label conditions were generally about 55 and 22% respectively. Standards of ^{3}H and ^{14}C toluene (Packard Co.) were also counted along with each batch of assay in order to evaluate such efficiencies as well as to permit the calculations in d.p.m.

No quenching correction was necessary since the external standardization showed no quenching in the samples relative to the pure standards. Background counts were not more than 12 c.p.m. in the ^{3}H channel and 14 c.p.m. in the ^{14}C channel. The temperature of the counter was at 4 - 5°C..

Under the counting settings employed, ^{3}H counts appearing in the ^{14}C channel were about 0.1-0.2%; ^{14}C counts entering ("spill") the ^{3}H channel was up to approximately 9 - 11% of those in the ^{14}C channel, and were corrected for by counting a pure ^{14}C standard with each batch of assays.

(f) Protein determination

In some of the experiments, estimation of the total protein in the preincubation media or homogenates was made by the Micro KJELDAHL method (277). This was carried out in Drs. N. DRUMMOND and R. SCRIVER's laboratories, Montreal Children's Hospital.

Preparations The digestion solution was made up of 10 gm. selenium oxide, and 10 gm. copper sulfate dissolved in a mixture of 750 ml. concentrated sulfuric acid (36N) and

250 ml. phosphoric acid.

Boric acid solution consisted of 40 gm. of boric acid, 200 ml. absolute ethanol, 10 ml. mixed indicator (0.066 gm. methyl red and 0.03 gm. bromocresol green dissolved in 100 ml. of 95% ethanol) made up to 1 litre with distilled water.

Aliquots of homogenates or the preincubation media were introduced into a Micro KJELDAHL digestor, set at maximum heating capacity, for 3 hours to convert the nitrogenous compounds to ammonium sulfate. The same digestion process was applied to the urea standards (range from 35 to 280 µg. nitrogen per ml.).

The content of the KJELDAHL flask was washed quantitatively with 3 x 1 ml. distilled water into the centre well of the Labanco Micro KJELDAHL distillation apparatus, to which 8 ml. of saturated sodium hydroxide solution were also added to create an alkaline condition necessary for the release of ammonia from the ammonium sulfate. During the distillation, the ammonia was trapped in a beaker containing 20 ml. (exact volume not essential) of boric acid solution. Distillation of ammonia was discontinued when the beaker contained 40 ml. of boric acid-distillate mixture. This should also be the moment when the intensity of the blue colour in the mixture was maximum.

The ammonia trapped in the boric acid solution was then titrated with $\frac{1}{70}$N/1000 sulfuric acid, to an end point identical to the light green colour of a blank which consisted of 20 ml. boric acid diluted to 40 ml. with distilled water.

The total protein content was taken as 6.25 times that of the total nitrogen values.

(B) Experimental

1. General Preincubation Procedure

(a) Preparation of tissues

Male hooded rats (Long-Evans strain), weighing 180 ± 20 gm., were anaesthetized with Nembutal (sodium pentobarbital) at a dose of 7 mg./100 gm. body weight, given intraperitoneally in the animal room. Dorsal bilateral adrenalectomy was performed. The excised glands were quickly trimmed free of surrounding fat on a crushed-ice surrounded Petri-dish, with caution of not damaging the glands. When the separation of the zones of the glands (i.e., the zona glomerulosa and the zona fasciculata-reticularis) was desired, the glands were carefully incised at the capsules and gently stroked horizontally to remove the internal zona fasciculata-reticularis (5). The capsules would contain the zona glomerulosa. The tissues were placed in 0.9% ice cold saline not more than two hours before use.

(b) Preincubation of the adrenal tissues (27)

The whole, quartered or zonated rat adrenal glands used for incubation or for preparation of a homogenate were always subjected to a preincubation period (unless otherwise stated)(27). The whole, quartered, or zonated glands were washed two times with 1 or 2 ml. of cold KRBG, gently blotted dry with filter paper to remove excess buffer, and quickly weighed on a microtorsion balance. The weighed tissues were placed in a beaker containing KRBG (generally 1 ml./15 mg. tissues). The preincubation took place in a DUBNOFF Metabolic Incubator, shaking at 60 cycles per minute, in 95% O_2 - 5% CO_2 atmosphere, for 1 hour at room temperature (2, 189). The preincubation media (PIM) were generally discarded at the end of the preincubation unless otherwise stated.

(c) Collection of the PIM and preincubated tissues for reincubation with labeled steroid precursor(s)

At the end of the preincubation described above, the PIM, when required, were kept and diluted with fresh KRBG for the re-incubation, the exact details were given in the experimental data of this Thesis.

The preincubated tissues were transferred to a fresh volume of KRBG medium to which labeled steroid precursor(s) had been dissolved for incubation.

(d) Preparation of adrenal homogenates

When a tissue homogenate was required, the adrenals, whether preincubated or not, were homogenized in an all-glass power-driven homogenizer for 1 minute in either cold 0.44 M sucrose (38) solution or KRBG (see Experiment II). The homogenization was performed in anice-bucket to minimize the alteration of the enzymic activity during the process.

(e) Preparation of sub-cellular particles (the mitochondria) (38, 41 - 43)

When the mitochondrial fraction was desired for study, the homogenates prepared with 0.44 M sucrose were centrifuged in a refrigerated automatic centrifuge (Servall RC 2B) at 2,500 r.p.m. (750 g) for 10 minutes. The supernatant was removed, with no washing of the nuclei, and re-centrifuged at 10,500 r.p.m. (13,300 g) for 10 minutes. During the centrifugation process and before the incubation, everything was kept cold in an ice-bath.

2. Incubation Conditions

The PIM, preincubated tissues, homogenates and the mitochondrial fractions of the rat adrenals were incubated from 10 minutes to 2 hours, depending on the experiment, but all in 95% O_2 - 5% CO_2, and generally at 37°C. with the Incubator shaking at 60 cycles per minute. The incubation medium was KRBG, including in the case of the mitochondrial fractions

obtained by centrifugation in 0.44 M sucrose.

3. Extraction of the Incubates

(A) At the end of the incubation

(i) Tissue incubation. When only the tissues were incubated, the supernatant medium was decanted and the tissues were washed with KRBG (1 ml.). This washing was added to the incubate. The enzymatic reaction in the incubates were stopped by the addition of 1 volume of acetone or dichloromethant with a thorough shaking. The tissues were homogenized in 3 ml. distilled water in a manner described in 1 (d) above in this section. This homogenate was either added to the supernatant (Experiment I) or processed separately (Experiment III).

(ii) Incubation of other kinds of preparations. When homogenates, PIM or sub-cellular fractions were incubated, the reactions were stopped with acetone or dichloromethane, either in the aliquots peridocially removed (Experiment II) or in the total media.

(B) Processing of incubates for either percent conversions or specific activity determinations of the steroids

Depending on the biological parameters studied, the incubates obtained were processed according to either or both of the following approaches.

(i) Specific activities determinations. When the the exogenous productions from the added precursor as well as the endogenous production from the in situ precursor was looked for, the incubates or the tissue homogenates were divided into two aliquots(Experiments I - III).

Aliquot II was processed for obtaining the specific activities of the steroids, i.e., after the evaporation of the acetone, the aliquot of the media was extracted with three volumes of dichloromethane. In the case of the tissue homogenates, a partition with petroleum ether (b.p. 30 - 60°C.) and 25% ethanol was carried out. The lipid extracts were acetylated with a labeled acetic anhydride (generally the tritiated type) of known specific activity (50 mc/mM, Chapter III, 5) after extraction of the acetates (78)and addition of non-radioactive ("cold carriers") carriers of the steroid acetates under study (e.g., aldosterone, corticosterone, 11-deoxycorticosterone and progesterone). The extract was applied on TLC (Figure 7 and Table I), eluted and processed as described in the later part of this section (page 74) for individual steroids until the constancy of $^{14}C/^{3}H$ ratios were reached.

(ii) Percent conversions. When only the percent conversion of the steroid produced from the labeled precursor added was required, as was generally the case for the

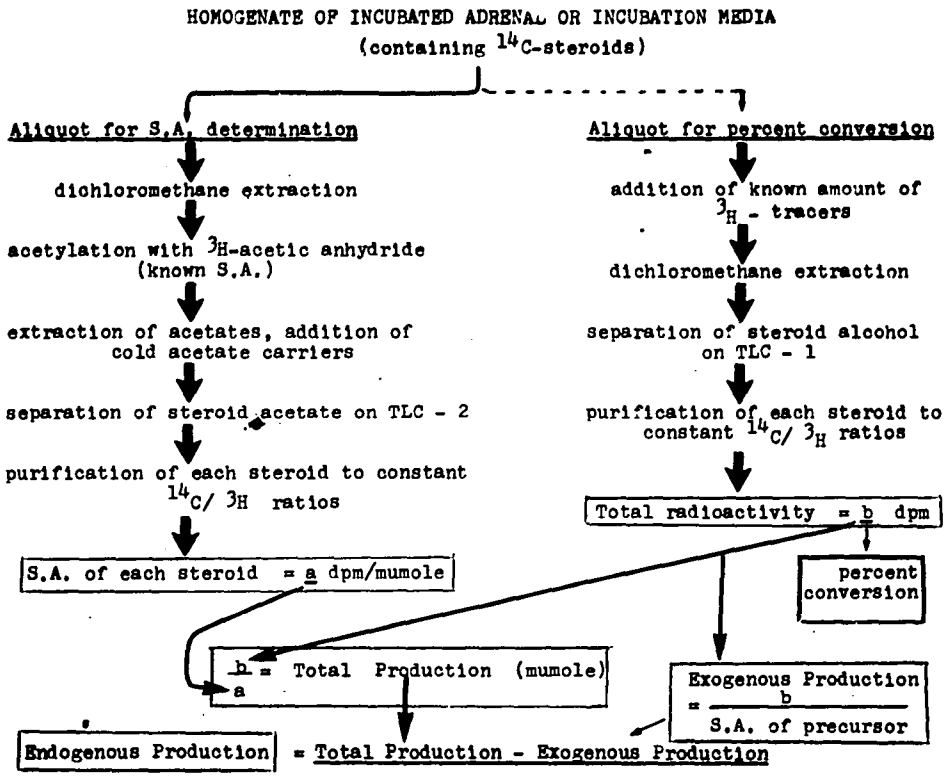


Fig. 6 Outline of the processing of adrenal incubates for the simultaneous measurement of exogenous and endogenous productions (mumoles).

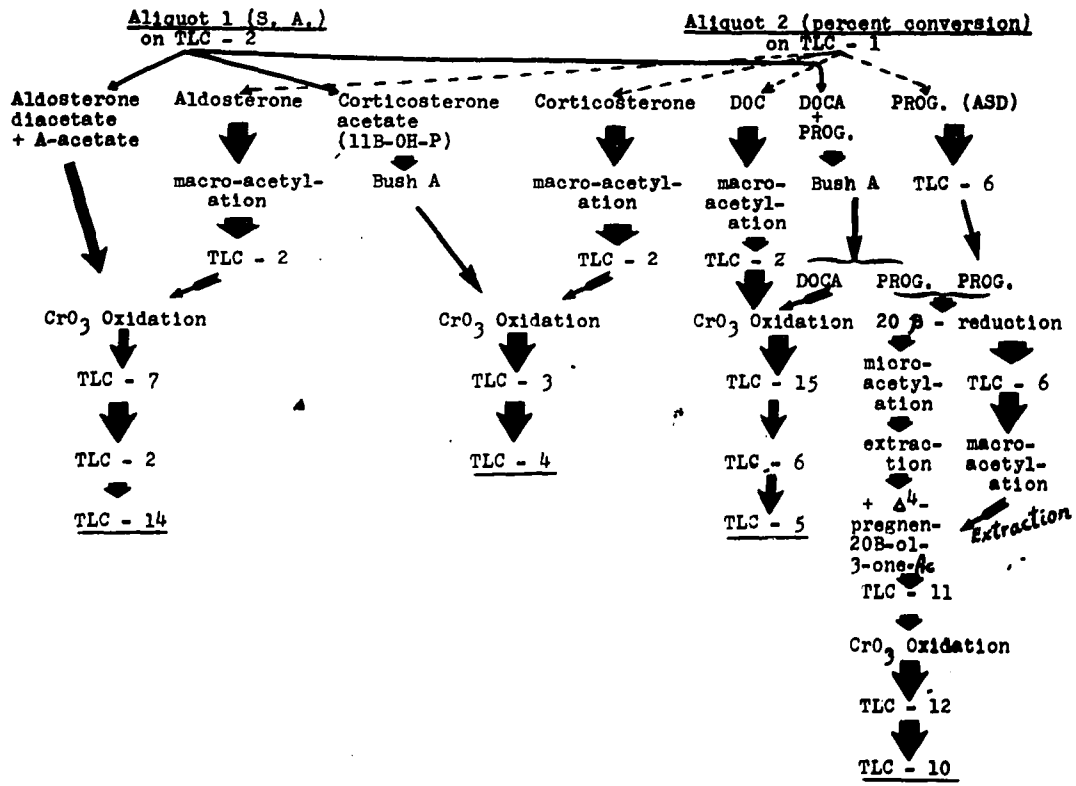


Fig. 7 Schedule of TLC, PC and chemical modifications of steroids from incubates.

subcellular fractions, PIM, diluted homogenate, where the endogenous production was deemed to be very small and not measurable, the incubates were directly extracted (as described in the previous paragraph) after the addition of tritiated steroid tracers (e.g., progesterone, aldosterone, corticosterone and 11-deoxycorticosterone) of known amounts as well as the internal (i.e. non-radioactive) steroid carriers for the recovery purposes. This applied also to aliquot II derived from (i) above.

The dichloromethane extracts were then developed on TLC as shown in Figure 7. The steroid free alcohol were thus processed individually to constant $^{14}C/^{3}H$ ratios through the formation of their acetates and their chromium trioxide oxidation products.

4. Special Notes on the Processing of Incubates

In the experiments with dilute media, PIM or mitochondrial fractions (see Experiments I - V, Chapter V), only the percent conversions of the precursors were examined. Hence, the total extract was processed.

In the experiments with tissues or concentrated homogenates, the specific activities as well as the percent conversions of the precursors and their products were analyzed, and therefore the incubates were divided into two known aliquots.

In Experiment III, there was not only the processing of

the media, but also the separation of the incubation system into tissues and media, for the analyses of both percent-conversion values and the specific activities of the steroids.

5. Systematic Processing of Aliquot I and Aliquot II of the Dichloromethane Extracts (Figure 7)

The following is a more detailed description of the systematic processing of the two aliquots.

(a) Aliquot I for percent conversion

Both the relevant ^{3}H-labeled steroid tracers (aldosterone, 11-deoxycorticosterone, corticosterone, and progesterone) of known and appropriate radioactivity (d.p.m.) and the corresponding non-radioactive ("cold") internal standards (approximately 20 µg. each) were added to a known portion (Aliquot I) of the dichloromethane extract. The extracts of aliquot I were quantitatively applied to TLC - 1.

For illustration, Figure 12 (ii) (Experiment III) is a photocopy of the radioautogram showing the typical and major radioactive metabolites, isolated from aliquot I, with 4-C^{14}-progesterone and 4-C^{14}-11-deoxycorticosterone as precursors incubated with whole and quartered rat adrenals, under the conditions described in the Experimental of Experiment III.

In order of decreasing polarity, radioactive spots running with internal standards or expected R_f are: 18-hydroxycorticosterone (18-OH-B), aldosterone, 18-hydroxy, 11-deoxycorticosterone (18-OH-DOC), corticosterone, 11β-hydroxyprogesterone (11β-OH-P), 11-deoxycorticosterone (DOC) and progesterone (Prog.). They were eluted with ethyl acetate-ethanol (3:2 v/v), and known aliquots of the samples were taken for counting. Further purification and identification of these fractions are given below:

(i) 18-Hydroxycorticosterone and 18-hydroxy,11-deoxycorticosterone

These two fractions were measured by U.V. absorption directly after TLC - 1. An aliquot was taken for counting for each of these compounds and the remaining portions were oxidized by periodic acid (34). After the extraction from this oxidation with dichloromethane, the dried extract was applied to TLC - 2 and the whole lot was eluted for final counting to check the agreement with the first aliquot counting. If sufficient quantities were available, a portion of these eluates were reacted with INH reagent for a check on quantification. Lack of available authentic standards prevented further purification of these two compounds.

(ii) Aldosterone fraction

This fraction was acetylated with non-radioactive acetic anhydride for 36 hours to yield aldosterone-18-21-diacetate (aldo-diacetate). The acetylated fraction was applied to

applied to TLC - 2. In this system, the R_f for aldosterone diacetate was 0.34. An aliquot from the aldosterone diacetate eluate was taken for counting, the remaining part was dried, and oxidized with chromium trioxide. By such operations, aldosterone diacetate gave aldosterone-11,18-γ-lactone-21-monoacetate (279). The oxidation product was chromatographed in TLC - 7. The aldosterone-11,18-γ-lactone-21-monoacetate fraction which had an R_f of 0.14 in this system was eluted, an aliquot taken for counting and the rest re-applied in TLC - 2, in which 11,18-γ-lactone-21-monoacetate had an R_f of 0.23. A constant $^{14}C/^{3}H$ ratio was usually attained after these chromatographies. TLC - 14, in which the monoacetate of aldosterone was also applied when the $^{14}C/^{3}H$ ratios were not yet constant, was the last TLC system in the schedule for aldosterone fraction and gave an R_f of 0.28 for its monoacetate.

(iii) Corticosterone fraction

This fraction was acetylated with non-radioactive acetic anhydride. The acetylated product, was re-applied to TLC - 2. The acetylated product running with internal pilot corticosterone-acetate (R_f = 0.19 was eluted, an aliquot taken for counting, the remaining thoroughly dried. The corticosterone-acetate (B-acetate) was oxidixed to 11-dehydrocorticosterone-21-acetate (A-acetate) by chromium

trioxide.

The oxidized product (A-acetate) was re-chromatographed in TLC - 3. In this system, the R_f for A-acetate was 0.35 and that of B-acetate was 0.25. The A-acetate fraction in TLC - 3 was eluted, with an aliquot taken for counting and the rest re-applied in TLC - 4, the fourth and final system for the fraction, which had an R_f 0.23 for the A-acetate. After elution, an aliquot was taken for counting. A constant $^{14}C/^{3}H$ ratio was generally achieved from the second chromatography onwards. This constant ratio served as a criterion for the identity of the ^{14}C-labeled product with ^{3}H-labeled corticosterone tracer.

(iv) <u>11-deoxycorticosterone fraction</u>

This fraction was acetylated with non-radioactive acetic anhydride ("macro"-acetylation) to convert it to 11-deoxycorticosterone-21-acetate (DOCA). An aliquot was taken for counting after its TLC - 1 (DOC R_f = 0.66) before the acetylation. The extracted DOCA from the acetylation mixture were applied to TLC - 2 where it showed an R_f of 0.46. After elution and aliquot taken for counting, the DOCA fraction was then oxidized with chromium trioxide to eliminate contaminants, if any, susceptible to chromium trioxide oxidation. The dichloromethane extract from the oxidation was re-chromatographed in TLC 15 (R_f = 0.40),

TLC - 6 (R_f for DOCA = 0.50) and TLC - 5 (R_f for DOCA = 0.34), an aliquot being taken for counting after each chromatography, until the constancy of $^{14}C/^{3}H$ ratio was attained.

(v) Progesterone fraction

Virtually all the ^{14}C-labeled materials in this fraction were unconverted 4-C^{14}-progesterone which had been added as an exogenous steroid precursor. This fraction was applied to TLC - 6 (R_f of progesterone = 0.54) and an aliquot of the eluate was taken for counting before the rest was enzymatically reduced to its 20β-ol form (see (A) 2 (c) of the present Chapter), 20β-ol-pregn-4-en-3-one, the dichloromethane extract of which was applied to TLC - 6 again. Besides taking the aliquot of its eluate for counting, it was followed by the acetylation (macro-) with non-radioactive acetic anhydride, extraction and the addition of 20β-acetoxy-pregn-4-en-3-one (20 µg.). TLC - 11 (R_f of this acetate = 0.32) followed but preceded the chromium trioxide oxidation. The dichloromethane extract of the oxidation mixture was applied to TLC - 12 (R_f = 0.28) and TLC - 10 (R_f = 0.27) after taking the aliquot for counting from each eluate. The TLC purification proceeded until constant $^{14}C/^{3}H$ was reached.

(vi) 11 -Hydroxyprogesterone fraction

Due to the unavailability of ^{3}H-labeled 11β-hydroxy-progesterone, this fraction was analyzed immediately after

TLC - 6 (R_f = 0.38), where the 11-dehydrocorticosterone were well separated from this compound (Figure 9), with the correction for losses. However, when the quantity of radioactivity of 11β-hydroxyprogesterone permitted, further TLC and chemical treatments similar to those for progesterone described above could be followed.

The details of a partial characterization of this fraction had been published by TSANG et al. (2).

(b) Aliquot II for specific activity determinations

The dichloromethane extract of this aliquot was acetylated with ^{3}H-labeled acetic anhydride of known specific activity. The method of acetylation involving a labeled acetic anhydride ("micro"-acetylation) (section (A) 3 (d) of this Chapter) had been previously described. After the extraction, 20 µg. of each of the following non-radioactive internal pilots: corticosterone-21-monoacetate (B-acetate), aldosterone-18,21-diacetate (aldo-diacetate), and 11-deoxycorticosterone-21-monoacetate (DOCA) were added to the dichloromethane extract, which was quantitatively applied to TLC - 2.

In this system, aldosterone diacetate and 11-dehydrocorticosterone acetate had an R_f of 0.34, B-acetate and 11β-OH-P had the same R_f of 0.19 while DOCA (R_f = 0.46) and progesterone (R_f = 0.56) were only partially separated. The

further separation and purification of these fractions were as follows.

(i) Aldosterone diacetate fraction. The oxidation of aldosterone 18,21-diacetate to aldosterone-11,18-γ-lactone-21-monoacetate, and the subsequent chromatography systems followed exactly the procedure described in Aliquot I for aldosterone diacetate. The aldosterone diacetate was separated from the A-acetate in TLC - 2 after the chromium trioxide oxidation.

A constant $^{14}C/^{3}H$ ratio was reached in the last two chromatographies after the oxidation by chromium trioxide.

(ii) Corticosterone acetate and 11B-hydroxyprogesterone fraction. This eluted fraction was re-chromatographed in Bush A for 36 hours. By this operation, the corticosterone acetate and 11β-hydroxyprogesterone were well separated (R_f 0.40 and 0.69 respectively).

The eluates of these two fractions from the scanned papergrams were dried and proceeded as that described in Aliquot I for corticosterone-acetate fraction, except for 11B-hydroxyprogesterone, which, due to experimental accident, was not processed as intended. A constant $^{14}C/^{3}H$ ratio was usually attained after the oxidation by chromium trioxide.

(iii) 11-deoxycorticosterone acetate and progesterone fraction. Since these two steroids were not well separated in TLC - 2(R_f for DOCA = 0.46, progesterone = 0.56), they were

eluted together and run for 4 hours in Bush A system, in which DOCA had an R_f of 0.5 and progesterone, 0.76. Each fraction was then eluted and treated separately as described below:

DOCA fraction further purification was the same as that for DOCA in Aliquot I.

Progesterone fraction. It should be emphasized that in Aliquot II there had been no addition of non-radioactive internal carrier of progesterone. This fraction was enzymatically reduced with 20β-hydroxysteroid dehydrogenase to form 20β-hydroxyprogesterone under conditions described previously (see (A) 2 (c) of this Chapter).

The dichloromethane extract of the reduction product was chromatographed in TLC - 2 to remove any unreacted progesterone (R_f = 0.56 for progesterone) from 20B-hydroxyprogesterone (R_f = 0.28). The latter fraction was eluted, well dried and acetylated with ^{3}H-acetic anhydride. After extraction of the acetylated product, 20 ug. of the non-radioactive internal carrier 20β-acetoxy-pregn-4-en-3-one (20β-hydroxyprogesterone acetate) was added and the fraction developed two-dimensionally in TLC - 11, in which the internal carrier had an R_f of 0.32. After the elution and an aliquot taken for counting, it was dried and oxidized by chromium trioxide to remove any oxidizable contaminants. The extract of the oxidation was applied in TLC - 12 (R_f=0.28),

followed by elution and an aliquot taken for counting. The remaining aliquots were dried and applied in TLC - 10 (R_f=0.27), eluted and taken an aliquot for counting. The final system to be applied was TLC - 13 (R_f = 0.21), when necessary, or, the systems after the chromium trioxide to be repeated, to achieve the final $^{14}C/^{3}H$ ratio constancy.

TABLE I

THIN-LAYER CHROMATOGRAPHY SYSTEMS

System	Solvent Composition (v/v)	Reference
TLC -1	Dichloromethane:Methanol:Water (150:10:1)	(280)
TLC -2	Chloroform : Acetone (9:1)	
TLC -3	Dichloromethane : Butyl Acetate (7:3)	
TLC -4	" : Acetone (9:1)	
TLC -5	Butyl acetate : Cyclohexane (1:1)	
TLC -6	Cyclohexane : Ethyl acetate (1:1)	(281)
TLC -7	Butyl acetate : Cyclohexane (10:1)	
TLC -8	Ethyl acetate : Cyclohexane (5:1)	
TLC -9	" : n-Hexane (1:1)	
TLC -10	Iso-octane : Dioxane (17:3)	
TLC -11	n-Hexane : Ethyl acetate (4:1)	
TLC -12	Toluene : Ethyl acetate (9:1)	(282)
TLC -13	Methanol : Toluene (1.5: 98.5)	
TLC -14	Acetone:Chloroform: Ethyl acetate (1:9:2)	
TLC -15	Chloroform : Butyl acetate (7:3)	
TLC -16	Dioxane : Iso-octane (3:7)	

TABLE II

PAPER CHROMATOGRAPHY SYSTEMS USED

System	Phase		Equilibration Time hrs.	Running Time hrs.	Reference
	Mobile	Stationary			
Bush A	Skellysolve C 5 :	Methanol-water 4 : 1	12	4	(283)
Bush 3	Skellysolve C-Benzene 667 : 333	Methanol-water 800 : 200	1	4-4½	(283)
T. P. G.*	Toluene	Propylene glycol -methanol	nil	varied with compound	(79)

* Systems of ZAFFARONI type which requires impregnation of the paper (stationary phase) but no equilibration time necessary

TABLE III

R_f VALUES OBTAINED IN DIFFERENT THIN-LAYER CHROMATOGRAPHY* SYSTEMS FOR THE STEROIDS STUDIED IN THIS THESIS
(* room temperature)

Steroid	S y s t e m s								
	1	2	3	4	5	6	7	14	15
Aldosterone	0.16								
" diacetate		0.34					0.25	0.37	
" 21-monoacetate		0.10							
" 11-18-γ-lactone-21-monoacetate		0.23					0.14	0.28	
Corticosterone	0.36	0.05				0.12			
" acetate		0.19	0.25	0.25					
A-acetate			0.35	0.23					
11-deoxy-corticosterone	0.66	0.24				0.34			0.27
" acetate		0.46			0.34	0.50			0.40

TABLE III (Cont'd.)

R_f VALUES OBTAINED IN DIFFERENT THIN-LAYER CHROMATOGRAPHY* SYSTEMS FOR THE STEROIDS STUDIED IN THIS THESIS

(* room temperature)

Steroid	Systems									
	1	2	6	8	9	10	11	12	13	16
Progesterone	0.8	0.56	0.54		0.63	0.22				
20β-Hydroxy-progesterone		0.28			0.48					
" acetate	. . . . .		0.54			0.27	0.32	0.28		
11β-Hydroxy-progesterone	0.58	0.20		0.38						
" (reduced)	. . . . .			0.22	0.12	0.02				
" acetate	. . . . .				0.34	0.34	0.59		0.13	0.47
11-keto-OH-P-acetate	. . . .	0.47				0.47				
18-Hydroxy-corticosterone	0.06									
" (HIO_4 oxid.)		0.09								
18-Hydroxy,11-deoxycortico-sterone	0.33									
" (HIO_4 oxid.)		0.35								

6. Calculation

(a) Conversion of radioactivity in c.p.m.(counts per minute) to d.p.m. (disintegrations per minute):

Assuming that after deducting the blank counts and spill overs of the ^{3}H or ^{14}C into the channel of the other, in the Scintillation Liquid Counter, the net counts of the standard labeled toluene (Packard Co.) (in either ^{3}H or ^{14}C forms) prepared in the laboratory at a known date with a known counts taken (allowing for radioactive decay, if applicable, and the specific gravity of toluene at the temperature of preparation) be Y, and the net counts of the toluene in the vial with respect to a given isotope (^{3}H or ^{14}C) on any other day subsequent to its preparation from the commercial batch (assuming negligible evaporation of toluene and well stoppered) be X counts,

the percent of machine efficiency with respect to this isotope concerned would be:

$$(X/ Y) \times 100$$

Let this be Q %.
It followed therefore the counts of a sample with, say U c.p.m., would have

$$U \times \frac{100}{Q} \text{ d.p.m.}$$

(b) Estimation of the percent conversion and production (mμmoles)from an exogenous radioactive precursor for any steroid

Arbitrarily, taking corticosterone as a conversion product, let

R_c = constant $^{14}C/^{3}H$ ratio of isolated ^{14}C-labeled corticosterone to ^{3}H-labeled corticosterone indicator added, with both isotopic counts in d.p.m.,

D_t = radioactivity (d.p.m.) of ^{3}H-corticosterone added to Aliquot I;

D_p = radioactivity (d.p.m.) of added ^{14}C-labeled steroid precursor.

n = fraction of total extract in Aliquot I

A_p = quantities (mμmoles) of added ^{14}C-labeled steroid precursor

Corrected radioactivity in ^{14}C-labeled corticosterone in <u>total</u> lipid extract of incubates:

$$= \frac{R_c \times D_t}{n} \text{ d.p.m.}$$

Percentage of the added ^{14}C-labeled precursor converted to corticosterone: $= \frac{R_c \times D_t}{n} \times \frac{1}{D_p} \times 100$

Quantities of ^{14}C-labeled corticosterone formed from the 4-C^{14}-precursor added (e.g., progesterone); i.e., exogenous productions: $\frac{R_c \times D_t}{n} \times \left(\frac{1}{\text{Specific activity of 4-C}^{14}\text{-precursor (d.p.m./mμmole)}} \right)$

(c) From the Aliquot II of the incubates (Experiments I - III), the calculations for the <u>specific activities</u> of isolated steroids would be as follows:

Taking corticosterone as an example again, let

S. A. = specific activity of ^{3}H-acetic anhydride (d.p.m./mμmole),

r_c = constant $^{14}C/^3H$ ratio of isolated doubly labeled

corticosterone-21-monoacetate.

Then the specific activity of corticosterone

$$= r_c \times \left(\frac{S.A.}{2}\right) \text{ d.p.m./mμmole}$$

(d) Computation of total endogenous productions of an isolated steroid product (Figure 6)

By dividing the total radioactivity (calculated in Aliquot I) of the ^{14}C-labeled steroid studied by its specific activity determined in Aliquot II, the total production (endogenous and exogenous conversion products) of the steroid could be obtained.

The total production (mμmoles) minus the exogenous production (mμmoles) coming from the conversion of the precursor (Aliquot I) gives the endogenous production (mμmoles) of the compound (Figure 6).

This method of the simultaneous measurement is sensitive and requires the exact measurement of the aliquot taken for the determination of the total radioactivity. The tracers added for the study of recovery have to be radiochemically pure (hence they were always re-chromatographed shortly before use).

CHAPTER V

RESULTS

Experiment I

Effects of the level of cellular organization of rat adrenal tissues and various factors on the 21-, 11β- and 18-hydroxylations.

The purpose of this experiment was to investigate the effects of the kinds of tissue preparations, the cofactors, the incubation time, the dilution, the substrate and enzyme concentrations on the hydroxylation of 4-C^{14}-progesterone by the zona glomerulosa and fasciculata-reticularis of the rat adrenal cortex.

The results of this experiment would serve as guide lines for the subsequent investigations, with respect to the heterogeneity of factors in adrenocorticoid biogenesis in vitro.

1. Experimental

The zona glomerulosa and fasciculata-reticularis of 60 adrenals of rats (180 - 200 gm. body weight) were separated (5) and preincubated separately for 1 hr. in KRBG

(30 mg. tissue/ml.) at room temperature (22°C.). The preincubation was carried out for 1 hr. (2) at room temperature since it has been reported that the preincubation media (PIM) enzymatic activities are markedly increased at lower temperatures (2, 189).

After the preincubation, the PIM was carefully decanted and the tissues were weighed. To the incubation vials containing 2 ml. of cold KRBG each, 15 mg. glomerulosa tissue was added to each of the vials G, H, I and 15 mg. fasciculata tissue was added to each of the vials 7, 8 and 9.

128 mg. glomerulosa tissue was homogenized in 8.5 ml. cold KRBG (15 mg./ml.), and 600 mg. fasciculata tissue was homogenized in 40 ml. cold KRBG (15 mg./ml.). This homogenate was diluted 12.5 times in some samples (2.4 mg. tissue/ml.) with cold KRBG.

The PIM, the glomerulosa and fasciculata slices, the dilute and concentrate homogenates were all incubated under the conditions indicated in Table IV, where the samples were shown to differ either in the addition of NADP plus G-6-P, malate, or the duration of the incubation, etc..

After the incubation per se, the incubates of samples

G, H, I, 7, 8 and 9 were separated into glands and media. The glandular tissues were homogenized in 1 ml. cold KRBG for each sample. These homogenates were combined with their respective media. Before the dichloromethane extraction, the incubates of samples G, H, I, J, K, 7, 8, 9, 10 and 11 were divided into two known aliquots. Aliquot I was for percent conversion and aliquot II for specific activity analyses (see Chapter IV (B) 3, 4 and Figure 6). The media relative to the PIM and to the dilute homogenate were not divided but taken whole for percent conversion analyses.

2. Results and Discussions

The percentages of conversion of 4-C^{14}-progesterone to aldosterone, 18-hydroxycorticosterone, 18-hydroxy,11-deoxy-corticosterone, 11-deoxycorticosterone, corticosterone and 11β-hydroxyprogesterone are given in Table V. Table VI summed up the <u>total</u> 21-, 11β- and 18-hydroxylation values of Table V for quick reference. By "total" hydroxylation at a given position of the steroid molecules, it is meant the <u>sum</u> of the percentages of hydroxylation (conversions) of 4-C^{14}-progesterone at that position of the steroid molecules of all different compounds analyzed.

For better comprehension, the results and discussions are presented as follows:

I. SLICE

Samples G and 7 were incubated with NADP, G-6-P and malate. Samples H and 8 were without the addition of NADP and G-6-P, while samples I and 9 were without malate added in their incubations (Table IV). Malate was added in accordance with the report of TALLAN et al. (181) on the action of malate in the 18-hydroxylation of steroids.

A. In Glomerulosa

(a) Percent conversion. As shown in Table V, the percent conversion of 4-C^{14}-progesterone (as indicated by the percent of 4-C^{14}-progesterone recovered) at the end of the incubation was low in sample H where no cofactors (NADP plus G-6-P) were added. However, according to Table VI (total percent hydroxylation of 4-C^{14}-progesterone), it can be seen that the total 21-hydroxylation (20.2%) was higher than either its total 11β- or 18-hydroxylation.

The addition of an NADPH-generating system (sample G), compared with H, increased in all the three (21-, 11β- and 18-) hydroxylations, but predominantly in the four-fold increase of the total 11β-hydroxylation of 4-C^{14}-progesterone (from 12.1 to 52.1%) and the three-fold increase of the total 18-hydroxylation (from 3.9 to 11.6%). The addition of malate in sample G besides the cofactors (NADP plus G-6-P) did not give a marked effect on the hydroxylation of 4-C^{14}-progesterone

other than a very slight inhibitory action on all the three aforesaid hydroxylations.

Turning to Table V, it can be seen that in sample H, 4-C^{14}-DOC, the 21-hydroxylated product of 4-C^{14}-progesterone, was formed in the highest proportion (10.4%). The percents of conversion to corticosterone and aldosterone were rather small, only 5.9 and 1.6% respectively. The addition of NADP plus G-6-P to the system greatly diminished the formation of DOC (0.2%, sample G) but, simultaneously, led to marked increases in the percents of conversion to corticosterone, aldosterone, 18-hydroxycorticosterone, 18-hydroxy,11-deoxy-corticosterone, 11-dehydrocorticosterone and 11β-hydroxy-progesterone. In short, all the compounds hydroxylated at either the 11β- or 18- position or both were increased by NADPH added. Thus, it can be concluded that <u>the action of NADPH was to increase the 11β- and 18-hydroxylation of progesterone in the slices</u>.

(b) Specific activities (Table VIII). Only the specific activities of progesterone, 11-deoxycorticosterone, corticosterone, 11-dehydrocorticosterone and aldosterone were obtained. It can be underlined that without the added NADPH (sample H), the specific activities were always much higher in all these compounds than those obtained in the presence of added NADPH, indicating the very small dilution with

endogenous precursor in the absence of added NADPH. For example, the specific activity of corticosterone in sample H was twice that of G, whose ratio of endogenous to exogenous production of corticosterone was 4 compared to 2 in H (Table VII). Although the addition of NADPH increased the endogenous production, yet the dilution of 4-C^{14}-progesterone with the endogenous production was still considerably smaller than those of other compounds under the same conditions. Thus, the specific activities of all other compounds were about two to three times lower than that of progesterone when NADPH was added. This could be due to the fact that the 4-C^{14}-progesterone added either did not mix readily with the endogenous progesterone due to its low solubility in the incubation fluid (162), or that progesterone is not the precursor of the steroids in the slices.

(c) Steroid productions (Table VII). The exogenous (external, from the added precursor), endogenous and total productions of progesterone, 11-deoxycorticosterone, corticosterone, 11-dehydrocorticosterone, and aldosterone are indicated in Table VII. In the absence of exogenous NADPH (sample H), the total production was very small for aldosterone and corticosterone, 0.2 and 0.8 mμmoles respectively. The quantities of the compounds generally related as precursors (progesterone and DOC) were relatively higher. With exogenous

NADPH (sample I) or NADPH and malate (sample G), the total productions of corticosterone and aldosterone were increased five to six times. This was due to a three-fold increase in the exogenous production and a seven-fold increase in the endogenous production. It can therefore be concluded that the effect of the NADPH-generating system on the glomerulosa slices was to increase the activity of the 11β- and 18-hydroxylations of progesterone by a factor of three to four. The effect on 21-hydroxylation was slight. There was also a much more marked increase in the endogenous production from endogenous precursor than from the 4-C^{14}-progesterone added. The effect of malate was not pronounced but somewhat inhibitory, when present, to the hydroxylation of progesterone.

B. In Fasciculata

(a) Percent of conversion. In the absence of exogenous cofactors (sample 8), the utilization of the added precursor (Table V) was higher than in Glomerulosa, since only 32.2% of progesterone was recovered in the system. The total 21-hydroxylation was twice the total 11β-hydroxylation, which, in turn, was five-fold the 18-hydroxylation activities in the same sample. In sample 8 (Table V), DOC was formed in almost the same proportion (21.4%) as corticosterone (20.6%). The DOC formed was negligible but the conversion to corticosterone was rather significant.

The presence of NADPH-generating system (sample 9) increased the utilization of progesterone, mostly for 11β-hydroxylation, which was increased by about three-fold, and 18-hydroxylation, which was enhanced approximately three times. There was only a raise of about 50% in the 21-hydroxylation activities.

The addition of malate to sample 7 with an NADPH-generating system did not affect significantly the total hydroxylations, except an approximately 50% increase in 18-hydroxylation.

(b) Specific activities (Table VIII). There were marked decreases in the specific activities of 11-deoxycorticosterone, corticosterone and 11-dehydrocorticosterone by a factor up to nine, as a result of great endogenous dilutions of these compounds in presence of the exogenous NADPH-generating system (Table VII). That of progesterone was essentially the same as its counterpart in glomerulosa, due to a small endogenous dilution.

(c) Steroid productions (Table VII). The total production of corticosterone with no cofactors added (sample 8) was 8 mμmoles, which was increased to about six-fold with NADPH. Such an increase was the result of a six- to seven-fold increase in the endogenous production. The presence of

malate stimulated the endogenous production significantly. It can be concluded here that the effect of NADPH-generating system on fasciculata slices was to increase the activity of the 11β- and 18-hydroxylation of progesterone and, to a lesser extent, the 21-hydroxylation also. It was accompanied by an even more marked increases in the endogenous precursors and their subsequent hydroxylations.

C. Glomerulosa versus Fasciculata Slices

The differences between the glomerulosa and fasciculata tissues without cofactors added (samples H and 8) are:

(a) The utlilzation of 4-C^{14}-progesterone added (Table V) was significantly higher by the fasciculata tissue.

(b) The conversion of 4-C^{14}-progesterone to aldosterone and 18-hydroxycorticosterone was found only in glomerulosa, which concomitantly accumulated much more 11β-hydroxyprogesterone (Table V) than fasciculata tissue. On the other hand, the conversion of added precursor to 18-hydroxy,11-deoxycorticosterone and corticosterone in the fasciculata tissue was three to six times higher.

(c) With a ten-fold endogenous production of corticosterone in fasciculata compared to the glomerulosa tissue, the specific activity of corticosterone in fasciculata was four times smaller than that of glomerulosa as a result of

malate stimulated the endogenous production significantly. It can be concluded here that the effect of NADPH-generating system on fasciculata slices was to increase the activity of the 11β- and 18-hydroxylation of progesterone and, to a lesser extent, the 21-hydroxylation also. It was accompanied by an even more marked increases in the endogenous precursors and their subsequent hydroxylations.

C. Glomerulosa versus Fasciculata Slices

The differences between the glomerulosa and fasciculata tissues without cofactors added (samples H and 8) are:

(a) The utlilzation of 4-C^{14}-progesterone added (Table V) was significantly higher by the fasciculata tissue.

(b) The conversion of 4-C^{14}-progesterone to aldosterone and 18-hydroxycorticosterone was found only in glomerulosa, which concomitantly accumulated much more 11β-hydroxyprogesterone (Table V) than fasciculata tissue. On the other hand, the conversion of added precursor to 18-hydroxy,11-deoxycorticosterone and corticosterone in the fasciculata tissue was three to six times higher.

(c) With a ten-fold endogenous production of corticosterone in fasciculata compared to the glomerulosa tissue, the specific activity of corticosterone in fasciculata was four times smaller than that of glomerulosa as a result of

greater endogenous dilution in the fasciculata tissue.

(d) The added NADPH exerted similar effects on both glomerulosa and fasciculata tissues, since in either case the conversion of 4-C^{14}-progesterone to corticosterone and 18-hydroxy,11-deoxycorticosterone were tripled, and that of 11β-hydroxyprogesterone were enhanced by a factor of seven to eight. The DOC was simultaneously decreased to insignificant amounts. Consequently, the action of added NADPH on the specific activities of the compounds in glomerulosa and fasciculata was similar, i.e., a general decrease by about 50%.

II. PIM

In the incubations of the PIM, NADP plus G-6-P were added to all the samples since without them there would be no hydroxylation activities. Samples A and 1 were without malate added; samples B and 2 incubated for only 30 minutes; samples C and 3 were references with NADP plus G-6-P and malate; samples D and 4 were diluted five times, samples E and 5 ten-times, both with KRBG, while samples F and 6 contained triple amounts of enzymes. Since the PIM can be considered as a very dilute homogenate (39), only the percent conversions of 4-C^{14}-progesterone were studied.

A. In Glomerulosa

Sample C is taken as reference. To this sample was

added NADP plus G-6-P and malate, which was found to increase the 18-hydroxylation of steroids (214). These media correspond to the preincubation of 7.5 mg. of rat adrenal tissue and contained 72 µg. protein/mg. tissue. The utilization of 4-C^{14}-progesterone by the PIM was very significant, since only 36.8% of it was recovered (Table V). In Table VI, it can be seen that the 11β-hydroxylation was the most pronounced (49%), followed by 21- (9.1%) and 18- (1.5%). Table V shows that the percent conversions to 18-hydroxycorticosterone, 11-deoxycorticosterone, 11-dehydrocorticosterone and corticosterone were very small and 11β-hydroxyprogesterone contributed most to the conversion products. The conversion to aldosterone was also very small (with difficulty in obtaining constant $^{14}C/^{3}H$ ratios due to the very low counts).

B. In Fasciculata

Sample 3 is taken as reference with added NADP plus G-6-P and malate. These media corresponded to 7.5 mg. of tissue and contained 34 µg. protein/mg. tissue. Again, the utliization of 4-C^{14}-progesterone was very significant, since only 24% of it was recovered (Table V). The order of importance of hydroxylation, similar to that of the glomerulosa, was 11β- (49.5%), 21- (14.3%) and 18- (2.2%). According to Table V, the most abundant conversion product was 11β-hydroxyprogesterone (37.4%).

C. Glomerulosa versus Fasciculata

There was not a very drastic difference in these two types of tissues with respect to their hydroxylation capacities at the 21-, 11β- and 18- positions of progesterone in the PIM. The 21- and 18-hydroxylations were slightly smaller in the glomerulosa. As indicated in Table V, 18-hydroxylation was mostly represented by 18-hydroxy,11-deoxycorticosterone in the fasciculata, but a mixture of 18-hydroxy,11-deoxycorticosterone, 18-hydroxycorticosterone and aldosterone in the glomerulosa. The production of corticosterone in fasciculata was significantly higher. Both types of media studied gave rise to an accumulation of 4-C^{14}-11β-hydroxyprogesterone. Enhancement of hydroxylation of progesterone to 11β-hydroxyprogesterone may be attributed to a relatively high concentration of progesterone or NADPH-generating systems (154,290), and to the inactivation of 21-hydroxylase (39).

D. Actions of the Different Factors

(1) Action of malate. The comparison of samples A and 1 (where no malate was added) with samples C and 3 (reference) shows that the presence of malate decreased by 50% the 21- and 18-hydroxylation, the 11β-hydroxylation being only slightly increased, due to a decrease in corticosterone, 11-dehydrocorticosterone and 18-hydroxy,11-deoxycorticosterone.

(2) Action of time. When samples C and 3 are compared with samples B and 2, the three-time increase in time of incubation led to an almost doubled utilization of 4-C^{14}-progesterone. However, Table VI shows that the 21- and 18-hydroxylations did not increase appreciably with time. The increase in 4-C^{14}-progesterone conversion was due to the increase in the 11β-hydroxylation. Thus, there was a huge increase of 11β-hydroxyprogesterone by a factor of two to three, but that of corticosterone was slight. In the PIM, the 21- and 18-hydroxylase systems were inactivated by prolonged incubation, but not the 11β-hydroxylase system.

(3) Effect of dilution. Samples D and 4, and E and 5 have been diluted five and ten times respectively with KRBG, compared to samples C and 3. The utilization of 4-C^{14}-progesterone was nearly halved when dilution took place (Table V), although the 21- and 18-hydroxylations were practically unaffected (Table VI). The striking phenomenon as a result of the dilution was the marked diminution in the 11β-hydroxylation, solely due to the decrease in the formation of 11β-hydroxyprogesterone. The decrease in the hydroxylation activities due to dilution could be the result of a greater dispersion of 4-C^{14}-progesterone in the medium, making it less available to the enzymes.

(4) Effect of increase in enzyme concentration.

Samples F and 6 contained three times as much of the initial PIM (1.5 ml.) than C and 3 (0.5 ml.). As expected, the utilization of 4-C^{14}-progesterone was greater in samples F and 6, which theoretically had three times as much enzymes as the reference samples C and 3. Despite the three-fold increase in 21- and five to six-fold increase in 18-hydroxylations, the 11β-hydroxylation was only slightly changed due to a lack of accumulation of 11β-hydroxyprogesterone. The overall effect of the increase in enzyme concentration was the enhancement of the 18-hydroxylations by a factor of two.

III. HOMOGENATE

Samples J and 10 are references, K and 11 had a 12.5-time dilution with KRBG, L and 12, M, 13, N and 14 all had a 12.5-time reduction of enzymes compared to samples J, K, 10 and 11. In addition, M and 13 had a 13-time reduction in 4-C^{14}-progesterone substrate, while N and 14 had their NADP and G-6-P reduced by 12.5 times. The aldosterone for the percent conversions were purified by periodic acid oxidations and only samples J, K, 10 and 11 were analyzed for the total production of steroids.

A. In Glomerulosa

(a) Percent conversion. Taking samples J as reference, it is conceivable that, in Table V, the utilization of 4-C^{14}-progesterone was rather exhaustive (only 3.8% recovered)

and, in Table VI, the total 21-, and 11β- and 18-hydroxylations did not differ significantly (around 40% mark). They were contributed mostly by the formation of corticosterone and 18-hydroxycorticosterone, besides a compound which behaved chromatographically like aldosterone before and after the periodic acid oxidation. The accumulation of 4-C^{14}-11β-hydroxy-progesterone was strikingly low (3.5%) and this applied to corticosterone (5.1%) also (Table V).

(b) Steroid productions (Table VII). Only the corticosterone productions were calculated. This was due to the fact that the ^{14}C and ^{3}H counts relative to aldosterone disappeared after the purification of the samples by acetylation and CrO_3 oxidation. It was uncertain if this was due to technical error or to the fact that there was no aldosterone in the samples, since the total production of corticosterone was rather small (2.2 mμmoles) even in sample J.

B. In Fasciculata

(a) Percent conversion. In sample 10, the conversion of 4-C^{14}-progesterone was also rather complete (8.3% recovered, Table V). Due to the formation of 18-hydroxy,11-deoxycorticosterone, the total 21-hydroxylation was slightly higher than the total 11β-hydroxylation, the total 18-hydroxylation (11%) being the smallest, only about one-fifth to one-sixth of either of the former hydroxylation activities. Corticosterone and

18-hydroxy,11-deoxycorticosterone accounted for most of the steroids converted from 4-C^{14}-progesterone; those contributions from 11β-hydroxyprogesterone and 11-deoxycorticosterone were negligible.

(b) Steroid productions (Table VIII). The total production of corticosterone was quite high (35.1 mμmoles) in sample 10.

C. Glomerulosa versus Fasciculata

The qualitative difference between the glomerulosa and fasciculata tissues was the formation of aldosterone and 18-hydroxycorticosterone only in the glomerulosa, besides the very low production and conversion to corticosterone in glomerulosa. The fasciculata, on the other hand, persistently showed much higher total 21-hydroxylation but an approximately seven-time smaller conversion to 11β-hydroxyprogesterone.

D. The Actions of the Different Factors

(1) Effect of the dilution of the homogenate. The samples K and 11 were diluted 12.5 times with KRBG, but J and 10 were not.

In the glomerulosa, the utilization of 4-C^{14}-progesterone was higher in the diluted homogenate (sample K). There was a slight decrease in the 21- and 18-hydroxylation (Table VI), whereas the 11β-hydroxylation of progesterone (Table V) was

enhanced thirteen times. This was the consequence of a diminution in 18-hydroxycorticosterone, aldosterone and 18-hydroxy,11-deoxycorticosterone, with a concomitant increase of corticosterone due to the increased 11β-hydroxylation. It is important to note that although the percent conversion to corticosterone was increased three times, its endogenous production was actually decreased (Table VII) two-fold (sample K). The specific activity of corticosterone (Table VIII) in sample K was thus four times higher than that of reference sample J with no dilution by KRBG.

In fasciculata samples 10 and 11, as for glomerulosa, the dilution with KRBG induced a higher utilization of 4-C^{14}-progesterone (Table V), with 0.7% (sample 11) and 8.3% (sample 10) of it recovered, indicating enhancement in the hydroxylation of progesterone. This was evidenced by the increases in the percent of conversion of 4-C^{14}-progesterone to 18-hydroxy,11-deoxycorticosterone, corticosterone and 11β-hydroxyprogesterone. Nevertheless, Table VII indicates a three-time decrease in the total production of corticosterone (sample 11), whose specific activity was four times that of reference sample 10. This was due to the much smaller endogenous dilution of the exogenously formed corticosterone in sample 11.

In both the glomerulosa and fasciculata homogenates, the 12.5-time dilution caused a diminution of the endogenous

precursor. Thus, the fact that 11β-hydroxylation in glomerulosa, and the 21-, 11β- and 18-hydroxylations of 4-C^{14}-progesterone in fasciculata were increased could be explained by the smaller endogenous dilution of the labeled precursor added, so that there would be a higher amount of radioactivity available to its subsequent conversion products. It must be emphasized that this does not mean a higher enzymatic activity, since the production of corticosterone was shown to be decreased three times in the fasciculata (sample 11) and slightly decreased in glomerulosa sample K (Table VII).

Two important differences in the effect of dilution of the glomerulosa and the fasciculata homogenate can be stated. Firstly, the dilution caused a high inactivation of the 18-hydroxylase system in the glomerulosa but not in the fasciculata (Table VI). This could explain the small difference in the total production of corticosterone between samples J (2.2 mμmoles) and K (2.0 mμmoles). Corticosterone which was not 18-hydroxylated would accumulate. Secondly, in the glomerulosa (sample K), the significant decrease in the total 21-hydroxylation (which was increased in fasciculata counterpart) plus the concomitant increase in the 11β-hydroxylation had caused a high accumulation of 11β-hydroxyprogesterone (46% sample K). It should be noted that this accumulation of 11β-hydroxyprogesterone in the glomerulosa bore the same ratio

to that of fasciculata with or without dilution by KRBG.

(2) Effect of the reduction of enzymes. Samples L is to be compared with K, and 12 with 11. Under the conditions of these samples, there was a decrease not only in the quantity of the enzymes in L and 12, but also in the amount of potential or endogenous precursor of the steroids. Samples L and 12, which theoretically contained 12.5 times less enzymes than K and 11, showed smaller utilization of the 4-C^{14}-progesterone (Table V) although not in proportion to their enzyme contents. It can be seen that, in Table VI, sample L had a two-fold decrease in the 21-, and three-fold decrease in the 18-hydroxylation activities, but an increase in 11β-hydroxylation of progesterone. This latter phenomenon was the consequence of the very high increase in 11β-hydroxyprogesterone accompanied by a decrease in all the 18-hydroxylated steroids as well as corticosterone (Table V), with the reduction of enzymes. Such decreases were, again, not proportional to the enzyme contents of L and 12. This was due in part to the greater saturation with the added substrate (4-C^{14}-progesterone) in L and 12 than in K and 11, since the endogenous precursor can be regarded as negligible in the latter.

(3) Effect of substrate reduction. When samples M and 13 are compared with L and 12, which initially had thirteen times as much 4-C^{14}-progesterone as M and 13, it can

be seen that the utilization of the precursor was slightly higher in M and 13, despite the rather similar total 21-, 11β- and 18-hydroxylation between all these four samples. The lack of an excess precursor at both levels tested could explain this observation, since the percents of hydroxylations (conversions) of 4-C^{14}-progesterone are, apparently, still on the ascending part of the substrate-velocity curve.

(4) Effect of the reduction of the cofactors. Sample N is to be compared with L, and 14 with 12. The 12.5-time reduction of cofactors in samples N and 14 caused a slight decrease in the utilization of 4-C^{14}-progesterone. This can be accounted for by the slight diminution of the 11β-hydroxylation. The reduction of cofactors led to two-fold increases in 21- and 18-hydroxylation (Table VI) in the fasciculata homogenate, but an insignificant decrease in the total 11β-hydroxylation in both types of the homogenates. These activities were the results of the increases in corticosterone and 18-hydroxy,11-deoxycorticosterone, as well as the decrease in the 11β-hydroxyprogesterone (Table V). In short, the higher cofactor concentration in glomerulosa (sample L) inhibited the 21- and 18-hydroxylation. This effect was less pronounced in the fasciculata homogenate. The 11β-hydroxyla- in both types of the homogenates, however, was scarcely affected by the higher cofactor concentration (154).

IV. CONCLUDING REMARKS

It can be concluded that the three preparations (PIM, slice and homogenate) possess the 21-, 11β- and 18-hydroxylation activities, but to different extents. Only the slices can hydroxylate the progesterone molecules without the addition of NADP plus G-6-P and, in this case, the 21-hydroxylation was the most active, followed by the 11β- and 18-hydroxylations. The PIM characteristically showed a high accumulation of 11β-hydroxyprogesterone, whereas the other steroids produced from 4-C^{14}-progesterone were much smaller. A peculiar phenomenon of the PIM is that the conversion of 4-C^{14}-progesterone to 11-dehydrocorticosterone was in higher quantities than in other preparations (Table V). In the glomerulosa, the conversion to aldosterone and 18-hydroxy-corticosterone by the dilute media____the PIM, and the diluted homogenate was always very poor; however, it was very high in the concentrate homogenate (samples J and K) and slices with added NADPH. In all cases, the 21-hydroxylation by the glomerulosa preparations was always much smaller than that of the fasciculata counterparts. In general, when NADPH was present, the glomerulosa 21-hydroxylation was usually smaller than its 11β-hydroxylation but greater than its 18-hydroxy-lation.

The results of this experiment demonstrated the difference in the hydroxylation of the added 4-C^{14}-progesterone

due to the different factors applied, reflecting that many factors can operate indirectly in an in vitro system. Consequently, it is of great importance to assess all these and other probable factors when interpreting such in vitro data.

It is apparent that different pools of enzymes and precursor exist, although it is difficult to ascertain their respective effects. Hence, in an incubation with slices, for example, it is essential to consider the endogenous production also, since it appears that it does not follow the same fluctuations as the exogenous production. In the present investigation, it can be seen that the exogenous and endogenous productions of a compound generally differ appreciably. An incubation with slices, in fact, can be considered as a system consisting of two pools of enzymes, one being the slice (tissue) itself and the other the media, which can be regarded as a very dilute homogenate or PIM (39). Both of these pools have different hydroxylation capacities. Thus, a higher hydroxylation of 4-C^{14}-progesterone merely represents a high exogenous production, and does not necessarily by itself mean a higher endogenous hydroxylase activity. Often the contrary can be observed, as illustrated by samples J, K, 10 and 11 (Table VII).

TABLE IV

INCUBATION CONDITIONS OF EXPERIMENT I
37°C.

P. = Progesterone

Sample No. Glom.	Fasc.	Tissue State	ml. PIM	Wt. of Tissue (mg.)	Vol. KRBG (ml.)	NADP (umoles)	G-6-P (umoles)	Malate	C^{14}-P. Precursor (d.p.m.)	Incub. Time (Hrs.)	Factor
A	1	PIM	0.5		1.5	2.4	6.7	Nil	749,200	2	No Malate
B	2	"	"		"	"	"	74	"	½	Incub. Time
C	3	"	"		"	"	"	"	"	2	Reference
D	4	"	"		7.5	"	"	"	"	"	Dilution (5x)
E	5	"	"		15.5	"	"	"	"	"	Dilution (10x)
F	6	"	1.5		0.5	"	"	"	"	"	Enzyme Increase (3x)
G	7	Slice		15	2	"	"	"	"	"	Reference
H	8	"		"	"	Nil	Nil	"	"	"	No Cofactors
I	9	"		"	"	2.4	6.7	Nil	"	"	No Malate
J	10	Homogenate		30	"	"	"	74	"	"	Reference
K	11	"		"	25	"	"	"	"	"	Dilution (12½x)
L	12	"		2.4	2	"	"	"	"	"	Reduced Enzyme (12½x)
M	13	"		"	"	"	"	"	68,220	"	Reduced Substrate (13x)
N	14	"		"	"	0.19	0.54	"	749,200	"	Reduced Cofactors (12½x)

TABLE V

PERCENT CONVERSION OF 4-C^{14}-PROGESTERONE TO DIFFERENT STEROIDS BY PIM, SLICE AND HOMOGENATE OF GLOMERULOSA (Gl) AND FASCICULATA (Fa)

Tissue State	Sample No.		C^{14}-P. Recov.		DOC		11β-OH-P		Cpd. B		Cpd. A		18-OH-DOC		18-OH-B		Aldo.
	Gl	Fa	Gl	Fa	Gl	Fa	Gl	Fa	Gl	Fa	Gl	Fa	Gl	Fa	Gl	Fa	Gl
PIM	A	1	39.3	32.5	1.2	1.2	28.7	22.6	10.2	16.4	4.2	5.1	2.4	4.3	0.19	-	0.2*
	B	2	69	56.2	-	0.2	11.8	16.7	3.1	8.4	3.2	2.2	0.6	2.1	0.03	-	0.02*
	C	3	36.8	24.6	0.6	-	41.6	37.4	4.7	9.9	2.3	2.2	1.1	2.2	0.16	-	0.2*
	D	4	60.2	53.4	0.7	0.5	16.5	16.6	6.6	11.3	2.2	2.4	1.2	2.4	0.05	-	0.1*
	E	5	58.1	54.9	1	-	15.7	14.7	6.7	12	2.6	2.6	1.3	3.8	0.07	-	0.1*
	F	6	8.4	18	-	-	36	10.2	15.4	25.7	8	4.8	5.8	14.7	0.58	-	0.8*
Slice	G	7	14.2	12.1	0.2	0.1	17.6	7.9	15.2	61	0.11	0.01	2.4	12.8	4.9	0.9	4.3
	H	8	60.6	32.3	10.4	21.4	3.4	1.1	5.9	20.6	0.02	0.03	1.1	4.1	1.2	0.1	1.6
	I	9	16.4	9.5	0.4	0.1	24.2	11.4	19	58.9	0.12	0.02	3.4	8	4.8	1	5.8
Homo-genate	J	10	3.8	8.3	0.5	-	3.5	0.5	5.1	52.8	0.4	0.16	7.2	11	16.3	-	16.3**
	K	11	0.9	0.7	-	-	46	6.6	18.1	76.7	1.1	0.05	4.6	14	3.5	-	3**
	L	12	14.2	4.4	0.1	-	67.3	16.6	10.6	43.6	0.5	0.04	3.2	10.9	0.3	-	0.4
	M	13	6.3	8.6	-	-	60.4	11.4	7.5	52	2.3	0.16	1.4	12.3	0.9	-	2*
	N	14	17.8	9.3	0.2	-	52.4	7.3	21.7	50	0.7	0.07	4.6	18.4	1.1	-	0.9

Incubation conditions are given in Table IV. (see Legend on following page)

LEGENDS TO TABLE V

Recov.	recovered
*	insufficient quantity to reach a strictly constant $^{14}C/^{3}H$ ratio
**	despite the constant $^{14}/^{3}H$ ratios reached for the percent conversion analyses, the ^{14}C and ^{3}H in the specific activity analyses disappeared

TABLE VI

TOTAL PERCENT HYDROXYLATIONS OF 4-C^{14}-PROGESTERONE BY PIM, SLICE AND HOMOGENATE OF GLOMERULOSA (Gl) AND FASCICULATA (Fa)

Tissue State	Sample	No.	Total Percent Hydroxylation					
			21-		11β-		18-	
	Gl	Fa	Gl	Fa	Gl	Fa	Gl	Fa
	A	1	18.4	27	43.5	44.1	2.8	4.3
	B	2	7	12.9	18.2	27.3	0.7	2.1
PIM	C	3	9.1	14.3	49	49.5	1.5	2.2
	D	4	10.9	16.6	25.5	30.3	1.4	2.4
	E	5	11.8	18.4	25.2	29.3	1.5	3.8
	F	6	30.6	45.2	60.8	40.7	7.2	14.7
	G	7	27.1	74.7	52.1	69.8	11.6	13.7
Slice	H	8	20.2	46.2	12.1	21.8	3.9	4.2
	I	9	33.5	68	53.9	71.3	14	9
	J	10	45.8	64	41.6	53.5	39.8	11
	K	11	30.3	90.8	71.7	83.4	11.1	14
Homo-	L	12	15.1	54.5	79.1	60.2	3.9	10.9
genate	M	13	14.1	64.5	72.6	63.6	4.3	12.3
	N	14	29.2	68.5	76.8	57.4	6.6	18.4

Incubation conditions are given in Table IV.

TABLE VII

STEROID PRODUCTIONS (mumoles/15 mg.tissue) IN DIFFERENT MEDIA BY SLICE AND HOMOGENATE OF GLOMERULOSA AND FASCICULATA

Tissue State	Sample No. Gl	Sample No. Fa	Progesterone Ex P	Progesterone En P	Progesterone T P	DOC Ex P	DOC En P	DOC T P	Corticosterone Ex P	Corticosterone En P	Corticosterone T P	11-dehydro-Corticosterone Ex P	11-dehydro-Corticosterone En P	11-dehydro-Corticosterone T P	Aldosterone Ex P	Aldosterone En P	Aldosterone T P
Slice	G		1	0.8	1.8	0.01	0.07	0.08	0.9	3.5	4.4	0.006	0.026	0.032	0.24	0.76	1
	H		3.6	0.7	4.3	0.7	0.3	1	0.3	0.5	0.8	0.001	0.003	0.004	0.1	0.1	0.2
	I		0.8	0.8	1.6	0.03	0.1	0.13	1.1	2.8	3.9	0.007	0.021	0.028	0.38	0.92	1.3
		7	0.6	0.3	0.9	0.01	0.11	0.12	3.6	46.9	50.5	0.001	0.001	0.012	-	-	-
		8	1.9	0.4	2.3	1.3	0.6	1.9	1.2	6.8	8	0.002	0.01	0.012	-	-	-
		9	0.7	0.4	1.1	0.01	0.04	0.05	3.5	35.1	38.6	0.001	0.018	0.019	-	-	-
Homo-genate	J		-	-	-	-	-	-	0.3	1.9	2.2	-	-	-	-	-	-
	K		-	-	-	-	-	-	1.1	0.9	2	-	-	-	-	-	-
		10	-	-	-	-	-	-	3.1	32	35.1	-	-	-	-	-	-
		11	-	-	-	-	-	-	4.5	7.7	12.2	-	-	-	-	-	-

Legends:

Ex P = Exogenous Production
En P = Endogenous Production
T P = Total Production

Gl = Glomerulosa
Fa = Fasciculata

Incubation conditions are given in Table IV.

TABLE VIII

SPECIFIC ACTIVITIES (d.p.m./mumole) OF THE STEROIDS ISOLATED FROM SLICE AND HOMOGENATE OF GLOMERULOSA (Gl) AND FASCICULATA (Fa)

Sample No.		Progesterone		11-deoxy-Corticosterone		Cortico-sterone		11-dehydro-Corticosterone		Aldo-sterone	Tissue State
Gl	Fa	Gl	Fa	Gl	Fa	Gl	Fa	Gl	Fa	Gl	
G	7	79,300	82,400	22,200	9,500	25,800	9,100	25,900	8,500	33,100	
H	8	105,200	104,900	83,900	86,600	52,200	19,300	30,200	16,600	61,000	Slice
I	9	71,300	80,100	26,600	17,400	35,900	11,400	32,100	11,000	34,500	
J	10	-	-	-	-	17,380	10,250	-	-	-	Homo-genate
K	11	-	-	-	-	69,820	47,170	-	-	-	

Initial specific activity of 4-C^{14}-progesterone used = 127,000 d.p.m./mumole.

Incubation conditions are shown in Table IV.

Experiment II

Effects of the concentration of NADP and G-6-P and incubation time on 21-, 11β- and 18-hydroxylations of the corticosteroid molecule by rat adrenal fasciculata tissue homogenates.

Since 21-, 11β- and 18-hydroxylations of progesterone by homogenates require reduced NADP as electron donor (220 - 223, 231, 234, 235) (Chapter II, 4 D (a)(ii), it is of interest to study the effect of the concentrations of exogenous NADPH-generating system on these different hydroxylations in a homogenate, and to investigate if they all require the same optimal concentrations, since a high concentration of NADPH was found to inhibit the 21-hydroxylase activity (SHEPPARD et al. (188)), and stimulated 11β-hydroxy-progesterone production from progesterone in rat adrenal homogenate (MAKOFF et al. (154)).

1. Experimental

The homogenate of the rat adrenal fasciculata tissue prepared in Experiment I was used in this experiment.

After defreezing at room temperature, 3 ml. portions of this homogenate (15 mg. tissue/ml.) was pipetted to incubation vials I to VI (inclusive). 3 ml. of the same homogenate was

diluted ten times with KRBG (0.15 mg. tissue/ml.), and 3 ml. portions were pipetted to incubation vials a to f (inclusive). Each incubation vial contained 17.5 mumoles of the precursor 4-C^{14}-progesterone (specific activity 1.27 x 10^5 d.p.m. per mumole). The different concentrations of NADP and G-6-P used in each sample are shown in Table IX. The incubation was carried out at 37°C. in a Dubnoff incubator, with a 5% CO_2 in O_2 gas phase.

After 5 minutes, 1 ml. aliquot was removed from each incubation vial into separate tubes. The incubation was continued for another 10 minutes, after which another 1 ml. incubate was removed as before. The remaining contents were incubated for another 75 minutes. In all cases, the aliquots were collected and shaken in dichloromethane to terminate the reactions.

For the dilute homogenate samples a to f (inclusive), a direct processing for the percent conversion analyses (see Chapter IV (B) 3, 4 and Figure 6) after the addition of known radioactivities of tritiated progesterone, 11-deoxy-corticosterone and corticosterone was performed.

After drying the dichloromethane, the concentrate homogenate samples I to VI (inclusive) were divided into two parts as described previously (Chapter IV (B) 3, 4 and Figure 6).

TABLE IX

INCUBATION CONDITIONS

37°C.

Fasciculata Homogenate Conc.	Dil.	Cofactor Concentrations G-6-P + NADP Per 3 ml.
I	a	60.4 µmoles 23.2
II	b	6.04 " 2.32
III	c	604 mµmoles 232
IV	d	60.4 " 23.2
V	e	6.04 " 2.32
VI	f	nil nil

Concentrate (Conc.) fasciculata homogenate: 45 mg. tissue per vial

Dilute (Dil.) fasciculata homogenate: 4.5 mg. tissue/vial

4-C^{14}-progesterone: 2,225,000 d.p.m./vial, specific activity 1.27×10^5 d.p.m. per mµmole

All samples were incubated for 90 minutes, with 1 ml. of the incubate removed from each sample at the end of 5 and 15 minutes.

6). Aliquot I was processed for the percent conversion after the addition of known amounts of radioactivity of tritiated progesterone, 11-deoxycorticosterone and corticosterone. Since tritiated 11β-hydroxyprogesterone and 18-hydroxy,11-deoxycorticosterone were not available, the results for the two components are calculated at the end of the second chromatographies of the systems for 11β-hydroxyprogesterone (TLC - 6) and 18-hydroxy,11-deoxycorticosterone (TLC - 2), which had just been oxidized by periodic acid (34). A recovery of 40% (a mean of the recoveries for corticosterone and progesterone after two chromatographies) is assumed for the correction of losses incurred during the purification processes. Aliquot II was processed for the specific activities of progesterone, 11-deoxycorticosterone and corticosterone (Chapter IV (B) 3, 4 and Figure 6).

Originally, it was intended to process the 11β-hydroxyprogesterone for the specific activities also, after the enzymatic reduction of the 20-ketone group. However, due to experimental accident, such an attempt was unsuccessful.

The results indicated in Tables X - XIII represent only those samples where the constancy of $^{14}C/^{3}H$ ratios have been attained.

2. General Considerations

(a) Figure 8 is the photograph of the radioautographs (developed after 36 hrs. exposure to the TLC - 1 chromatoplates) showing the metabolites present in samples I to VI and a to f (inclusive) for the 5, 15 and 90 minutes incubations. In Figure 8, it can be seen that in the dilute homogenate samples a to f (in order of decreasing concentration of exogenous NADPH-generating system) there was always an excess of 4-C^{14}-progesterone, which was diminished significantly in samples b and c after 90 minutes. Sample a, with the highest cofactor concentration (60.4 µmoles G-6-P, 23.2 µmoles NADP) showed an inhibition of the hydroxylation of progesterone in the first 5 minutes. There was then no detectable radioactivity incorporated. The conversion of 4-C^{14}-progesterone began slowly after 15 minutes to form DOC and, at 90 minutes, corticosterone, 11-deoxycorticosterone and 11-dehydrocorticosterone appeared. These two latter compounds were well separated by TLC - 6, as shown in Figure 9. In samples b (6.04 µmoles G-6-P, 2.32 µmoles NADP) and c (604 mµmoles G-6-P, 232 mµmoles NADP) there were some conversion to DOC after 5 minutes. After 15 minutes, corticosterone, 18-hydroxy,11-deoxycorticosterone, 11β-hydroxyprogesterone with 11-dehydrocorticosterone were detected. They were increased after 90 minutes, but there was especially a marked increase in 11β-hydroxyprogesterone. Sample d (60.4 mµmoles G-6-P, 23.2 mµmoles NADP) yielded only DOC which was

accumulated with increased incubation time. In sample e (6.04 mμmoles G-6-P, 2.32 mμmoles NADP) and f (without cofactors added), there were no incorporation of 4-C^{14}-progesterone.

(b) In the concentrate homogenate samples I to VI (in order of decreasing concentration of exogenous NADPH-generating system), there was a considerable quantity of 4-C^{14}-progesterone remained in each sample, but most of it had been converted in 15 minutes in the presence of high concentrations of NADPH (samples I - III, 60.4 μmoles - 604 mμmoles G-6-P, 23.2 μmoles - 232 mμmoles NADP). At lower concentrations of the cofactors (or in the absence of), the precursor was always poorly utilized or totally unconverted. Sample I, with the highest cofactor concentration (60.4 μmoles G-6-P, 23.2 μmoles NADP) showed a remarkable conversion of the C^{14}-precursor to DOC, corticosterone, 18-hydroxy,11-deoxycorticosterone in 5 minutes. Thereafter, DOC was progressively decreased to almost nothing in 90 minutes, whereas there was a steady increase in corticosterone and 18-hydroxy,11-deoxycorticosterone. 11β-hydroxyprogesterone and 11-dehydrocorticosterone appeared only in 15 minutes and were increased markedly in 90 minutes. Sample II (6.04 μmoles G-6-P, 2.32 μmoles NADP) had no detectable DOC in any period of the incubation practically, although corticosterone,

18-hydroxy,11-deoxycorticosterone, 11-dehydrocorticosterone and 11β-hydroxyprogesterone were present in 5 minutes and increased during the following 10 minutes. Sample III (604 mμmoles G-6-P, 232 mμmoles NADP) yielded DOC, corticosterone, 18-hydroxy,11-deoxycorticosterone, 11-dehydrocorticosterone with 11β-hydroxyprogesterone in 5 minutes and considerable quantities of them were present even after 90 minutes. Sample IV (60.4 mμmoles G-6-P, 23.2 mμmoles NADP) gave rise only to DOC throughout the incubation. At the lowest cofactor concentration (sample V, 6.04 mμmoles G-6-P, 2.32 mμmoles NADP) or in the absence of the cofactors (sample VI, a control) there was no apparent conversion of 4-C^{14}-progesterone.

The quantitative as well as the qualitative differences of the metabolites of 4-C^{14}-progesterone as a function of the concentration of the NADPH-generating system, the length of incubation and the enzyme quantity shall be clarified by the study of the quantitative data obtained.

3. Results and Discussions

In this experiment, the enzyme and cofactor concentrations as well as the incubation time effects have been investigated. These parameters shall be considered separately. The results of the experiment are presented in Tables X - XIII and Figures 8 - 11. The results for samples V, VI, e and f are not indicated as there were no detectable conversion on

the radioautographs.

The percent conversions of progesterone into corticosterone, 18-hydroxycorticosterone, 11-deoxycorticosterone and 11β-hydroxyprogesterone, and percent progesterone recovered are given in Table X for the dilute homogenate (samples a to d) and Table XI for the concentrate homogenate (samples I to IV) of the fasciculata tissue.

A. Dilute Homogenate

It is noteworthy that the dilute homogenate in this experiment contained basically ten times less enzymes and other cellular elements besides the potential precursor, than the concentrate homogenate. There was also a ten-time dilution of the enzymes in the dilute homogenate. However, the quantity of 4-C^{14}-progesterone and exogenous cofactors were the same in the respective samples of both types of homogenates. With this information in mind, it can be deducted that the substrate saturation with the exogenous precursor would be much higher for the dilute homogenate. As in Experiment I, this latter implication would be responsible for a diminished endogenous production. This is the reason for the assumption that the metabolites formed in the dilute homogenate were mostly from the 4-C^{14}-progesterone precursor. In view of this phenomenon, no specific activity determinations for the dilute homogenate samples were carried out. In considering the effect

of duration of incubation, it is conceivable in Figure 8 that 4-C^{14}-progesterone was always in excess in all samples. Table X and, particularly, Figure 10 show the overall 21-, 11β- and 18-hydroxylations as a function of time.

The overall 21-hydroxylation of 4-C^{14}-progesterone is shown in Figure 10 (i) for samples a, b, c and d with total percent conversions plotted against incubation time. The 21-hydroxylation of progesterone was very similar and significant in samples b, c and d after 5 minutes, but very small in a. The increases in a thereafter was slow but constant. The slope of the increase for d was flattening after 5 minutes and for b, c after 15 minutes.

Looking at the concentrations of the cofactors used, the cofactor concentration of sample c seems to be optimal for 21-hydroxylations of progesterone, followed by those of b, d and a. However, with lowest cofactor concentration, sample e (6.04 mμmoles G-6-P, 2.32 mμmoles NADP),or without cofactors (sample f),there were no hydroxylations. In short, 21-hydroxylation took place with the minimum cofactor concentration of 60.4 mμmoles G-6-P and 23.2 mμmoles NADP (sample d), and optimal concentration of 604 mμmoles G-6-P and 232 mμmoles NADP (sample c). Above these concentrations (samples b and a), progressive inhibitions of hydroxylation of progesterone were seen.

Figure 10 (iii) indicated the overall 11β-hydroxylations of progesterone. In samples b and c only were 11β-hydroxylations detected throughout the incubation, for sample a it was only after 90 minutes. There was no 11β-hydroxylase activity at all in sample d. It is noteworthy that the 11β-hydroxylation seemed to be delayed in the first 5 minutes after which there was an abrupt rise. In terms of cofactor concentrations, samples b and c showed optimal activities, while those in samples d and e were insufficient to support any 11β-hydroxylation. Sample a, with the highest cofactor concentration of NADPH, showed both a delay as well as a marked inhibition of the 11β-hydroxylation of 4-C^{14}-progesterone.

The 18-hydroxylation of 4-C^{14}-progesterone (forming 18-hydroxy,11-deoxycorticosterone) is shown in Figure 10 (ii). 18-hydroxylations were detected throughout the incubation only in samples b and c; for samples a and d it was only 90 minutes. The maximal activity was under 4% conversion of 4-C^{14}-progesterone and for b and c, such activities were almost identical throughout the incubation, and were much smaller than the 11β- and 21-hydroxylations. It can be said that there was also a delay in 18-hydroxylation of progesterone.

In short, the hydroxylation of progesterone in the dilute homogenate occurred first at the 21-position. This was followed by 18-hydroxylation (which was always small and leveled off

rapidly) and 11β-hydroxylation, which showed a characteristic abrupt increase with time after the first 5 minutes. Considering the individual compounds shown in Table X, DOC was the only steroid produced in the first 5 minutes in practice. Thereafter, corticosterone, 18-hydroxy,11-deoxycorticosterone and 11β-hydroxyprogesterone were found in increasing amounts in samples b and c (see also Figure 9 (ii)), whereas DOC itself disappeared progressively. There was a three-fold increase in corticosterone between 15 and 90 minutes, and a six-fold increase in 11β-hydroxyprogesterone and 18-hydroxy, 11-deoxycorticosterone in the same period. Since 11β-hydroxylation was much more active than the 21-hydroxylation in these samples, 11β-hydroxyprogesterone also accumulated (30 -39% conversion). In sample a, corticosterone and 11β-hydroxyprogesterone appeared after 90 minutes (Figure 9 (ii), with a concomitant decrease in DOC. In sample d, DOC increased very significantly with time but there was no 11β-hydroxylation detected (Table X).

In conclusion, hydroxylation of progesterone to form DOC seems to be a prerequisite for the activity of 11β-hyroxylase system to give rise to corticosterone, since DOC disappeared when corticosterone was formed. Once the 11β-hydroxylase was activated, it dominated over the 21-hydroxylase activity, probably due to the steady activation of the 11β-

hydroxylase and inactivation of the 21-hydroxylase as the incubation time was prolonged (189), with the subsequent accumulation of 11β-hydroxyprogesterone. The 18-hydroxylation might require the initial 21-hydroxylation activity, as it was increased with a delay and in parallel to the increase of the 21-hydroxylation. 604 mμmoles G-6-P and 232 mμmoles NADP (sample c) was the optimal cofactor concentration for the overall 21-, 11β- and 18-hydroxylations, and inhibitions of these hydroxylases began with 6.04 μmoles G-6-P and 2.32 μmoles NADP (sample b).

C. Concentrate Homogenate

For the effect of the duration of incubation, Table XI shows that the percent of 4-C^{14}-progesterone recovered after 5 minutes for samples IV, III, II and I were 55, 26, 22 and 35 respectively, but after 15 minutes, there was almost no progesterone left, except for sample IV (51.2%). Hence, due to the "shortage" of the added 4-C^{14}-progesterone precursor, the results of the percent conversions as a function of time would not precisely reflect the activity of the hydroxylases in the incubation media. Indeed, from Table XI, it can be seen that the variations in the total 21-, 11β-hydroxylations between 15 and 90 minutes were slight, though somewhat more pronounced for the 18-hydroxylation.

The comparison of the percent conversion values between

the concentrate and dilute homogenates (Tables X and XI) at 5 minute incubation showed that the 21-, 11β- and 18-hydroxylation of 4-C^{14}-progesterone were much more marked in the concentrate homogenate. This would be expected to be even more marked since there was theoretically ten times more enzymes in the concentrate homogenate. A striking difference is that there was no delay between the 21- and 11β-hydroxylations in the incubation of the concentrate homogenate. In sample IV (60.4 mμmoles G-6-P, 23.2 mμmoles NADP) only, the DOC coming from 4-C^{14}-progesterone remained unchanged throughout the incubation, indicating the absence of the 11β-hydroxylase activity. In sample III (604 mμmoles G-6-P, 232 mμmoles NADP), the 11β-hydroxylation was activated and gave rise to corticosterone and some 11β-hydroxyprogesterone (Figure 9 (i)). However, the total 21-hydroxylation was (70.8% at 90 minutes) higher than the total 11β-hydroxylation (51.1% at 90 minutes). In sample II (6.04 μmoles G-6-P, 2.32 μmoles NADP), the total 11β-hydroxylation was only slightly higher than the 21-hydroxylation (Table XI).

As outlined under Experimental, an aliquot of the concentrate homogenate was processed to measure the specific activity of the steroid in the different extracts. Table XIII shows the specific activities (d.p.m./mμmole) of the steroids obtained. Their specific activities were calculated only when

the constancy of the $^{14}C/^{3}H$ ratios were reached. The specific activities of corticosterone in samples I, II and III showed that there were very important endogenous productions after 5 minutes in comparison with the specific activities of progesterone. During the 85 minutes that followed, there was a further, but less pronounced, dilution. It can be noted that the specific activities of progesterone were much greater than those of corticosterone, raising the question of progesterone being a precursor of corticosterone (158 - 160).

From the total radioactivities incorporated and the specific activities, it was possible to calculate the total production, endogenous and exogenous productions (Figure 6) (see Chapter IV (B) 6). The total production data would give a better understanding of the total hydroxylation activities in the system during the period of incubation. These results can be found in Table XII. The exogenous productions coming from 4-C^{14}-progesterone added did not vary appreciably with time or samples, but the total productions of corticosterone in samples I, II and III (60.4 - 0.604 µmoles G-6-P, 23.2 - 0.232 µmoles NADP) increased with time as shown in Figure 11. The rate of the increase tended to decrease with time. The productions in sample V (6.04 mµmoles G-6-P, 2.32 mµmoles NADP) and IV (60.4 mµmoles G-6-P, 23.2 mµmoles NADP) were determined directly on the samples using the double isotope derivative assay (95) but they were only in trace amounts

even after 90 minutes (hence data not shown). Starting with the cofactor concentration of sample III (604 mμmoles G-6-P, 232 mμmoles NADP) upwards, the production of corticosterone was appreciable. Corticosterone, as shown in Figure 11, was optimally produced in sample II (6.04 μmoles G-6-P, 2.32 μmoles NADP) but its production suffered from inhibition at a higher concentration of the cofactors (sample I, 60.4 μmoles G-6-P, 23.2 μmoles NADP).

D. Concentrate versus Dilute Homogenate

Two important differences between the concentrate and dilute homogenates can be drawn. Firstly, only in the case of the concentrate homogenate was the utilization of 4-C^{14}-progesterone exhaustive even in 15 minutes of incubation (about 3% recovered). This may be explained by the fact that although the substrate saturation was apparently greater in the dilute homogenate, there had been not only a ten-time decrease of the enzyme systems and the endogenous precursor, but also a simultaneous ten-time dilution of both. Thus, the enzymatic activities in the dilute homogenate were diminished accordingly (290). Another difference that can be cited was the "delay" of the 11β- and 18-hydroxylations of progesterone observed in the dilute homogenate alone.

In both dilute and concentrate homogenates, there was

an identical minimal concentration of cofactors required for the hydroxylation activities, and an optimal concentration for maximal activities. Higher cofactor concentration was inhibitory to the hydroxylation activities, especially in the dilute homogenate.

E. Concluding Remarks

The concentration of the cofactor plays a crucial role in the steroid productions by the concentrate as well as dilute homogenate. Too low (sample IV and d) a concentration permitted only the formation of DOC, which was not appreciably increased in quantity with time. At a higher concentration (sample III and c), the 11β-hydroxylation was activated, yielding corticosterone and 11β-hydroxyprogesterone. The rate of 11β-hydroxylation was not lower than that of 21-hydroxylation and DOC was still accumulated, at least in the first 15 minutes. As the cofactor concentration was further increased (sample II), there was not only no more accumulation of DOC, but increase in corticosterone was very pronounced. The 11β-hydroxylation activities were the highest of all and the production of 11β-hydroxyprogesterone was markedly increased. When the highest cofactor concentration was reached (sample I and a), impairment of the 11β- and 21-hydroxylations were observed.

TABLE X

PERCENT CONVERSION OF 4-C^{14}-PROGESTERONE TO DIFFERENT STEROIDS BY DILUTE FASCICULATA HOMOGENATE WITH VARIED NADPH CONCENTRATIONS AND INCUBATION TIMES

Sample	Incubation Time (mins.)	Prog. Recov.	Cpd. B	18-OH DOC	DOC	11β-OH-P	Total Hydroxylations 21-	Total Hydroxylations 11β-
	5	61	-	-	6	-	6	-
d	15	52.8	-	-	8.7	-	8.7	-
	90	51.2	-	0.45	20.1	-	20.6	-
	5	57	1.3	0.1	4.9	0.6	6.3	1.9
c	15	37.7	13.2	1.0	2.6	5.1	16.8	18.3
	90	14.9	39.1	3.7	0.6	30	13.4	69.1
	5	69	0.2	0.1	4.8	-	5.1	0.2
b	15	50.7	10.8	0.6	2.5	3.9	13.9	14.7
	90	7.9	31.2	3.4	0.4	39	35	70.2
	5	71	-	-	0.7	-	0.7	-
a	15	55.3	-	-	2.7	-	2.7	-
	90	53.3	10.8	1.6	2.0	8.2	14.4	19

Incubation conditions are given in Table IX.
Recov.=recovered

TABLE XI

PERCENT CONVERSION OF 4-C^{14}-PROGESTERONE TO DIFFERENT STEROIDS BY CONCENTRATE FASCICULATA HOMOGENATE WITH VARIED NADPH CONCENTRATIONS AND INCUBATION TIMES

Sample	Incubation Time (mins.)	Prog. Recov.	Cpd. B	18-OH DOC	DOC	11β-OH-P	Total Hydroxylations 21-	Total Hydroxylations 11β-
IV	5	54.5	-	-	7.3	-	7.3	-
	15	54.5	-	-	8.7	-	8.7	-
	90	51.2	-	-	11.6	-	11.6	-
III	5	26.4	18	6	8.2	4.7	32.2	22.7
	15	5.3	32.3	6.9	12.5	5.4	54.3	37.7
	90	3.2	44.8	4.5	15.4	6.3	70.8	51.1
II	5	21.9	21.1	4.1	1.1	7.2	26.3	28.3
	15	2.6	40.1	4.7	-	16.7	44.8	56.8
	90	0.3	49.1	6.	0.3	8.9	55.4	58
I	5	35.2	19.3	4.5	20.7	-	44.5	19.3
	15	3	41.7	6.4	3.2	0.3	51.3	42
	90	0.9	39.3	4.3	0.4	4.8	44	44.1

Incubation conditions are given in Table IX.

Recov. = recovered

TABLE XII

STEROID PRODUCTIONS (mμmoles) OF CONCENTRATE FASCICULATA HOMOGENATE WITH VARIED NADPH CONCENTRATIONS AND INCUBATION TIMES

Sample	Incubation Time (mins.)	11-deoxycorticosterone			Corticosterone		
		Ex P	En P	T P	Ex P	En P	T P
IV	5	1.7	1.08	2.78	-	-	-
	15	1	1.23	2.23	-	-	-
	90	1.3	2.46	3.76	-	-	-
III	5	1.4	2.9	4.3	3.2	11.8	14.9
	15	2.2	16.1	18.3	5.7	31.2	36.8
	90	2.7	21.4	24.1	7.8	43.6	51.4
II	5	0.2	0.7	1	3.7	19.5	23.1
	15	-	-	-	7	62.9	69.9
	90	-	-	-	8.6	164.7	173.3
I	5	3.6	6.4	10.1	3.4	26.1	29.4
	15	0.6	6.1	6.7	7.3	46.5	53.8
	90	-	-	-	6.9	109.8	116.7

Incubation conditions are shown in Table IX.

Legends:

Ex P = Exogenous Production

En P = Endogenous Production

T P = Total Production

$4\text{-}C^{14}$-progesterone precursor: 17.52 mμmoles added, specific activity 1.27×10^5 d.p.m. per mμmole

TABLE XIII

SPECIFIC ACTIVITIES (d.p.m./mμmole) OF STEROIDS PRODUCED BY CONCENTRATE FASCICULATA HOMOGENATE IN TABLE XII

Steroid	Incubation Time (mins.)	Samples				
		I	II	III	IV	V
Cortico-sterone	5	14,600	20,300	26,800	-	-
	15	17,200	12,800	19,600	-	-
	90	7,500	6,300	19,400	-	-
11-deoxy-cortico-sterone	5	45,800	28,800	42,400	69,500	-
	15	10,700	-	15,300	72,400	-
	90	-	-	14,200	68,500	-
Prog-esterone	5	70,100	37,800	49,100	113,700	117,600
	15	-	-	-	120,400	124,400
	90	-	-	-	120,100	115,600

4-C^{14}-progesterone precursor: specific activity 127,000 d.p.m./mμmole.

Fig. 8. Radioautograms taken of TLC chromatograms on TLC - 1 (Table I) (developed after 36 hrs. exposure) showing the incorporation of 4-C^{14}-progesterone into different steroids when incubated with dilute and concentrate fasciculata homogenates of rat adrenals under the conditions shown in Table IX. (Fig. 8 reduced scale)

(see following photograph)

Legends to Figure 8

Roman numerals	=	concentrate fasciculata homogenate sample numbers
small English letters	=	dilute fasciculata homogenate sample numbers
S. L. __	=	starting line (i.e., line of application on TLC chromatoplate)

For abbreviations of steroids in Figure 8, see page xii.

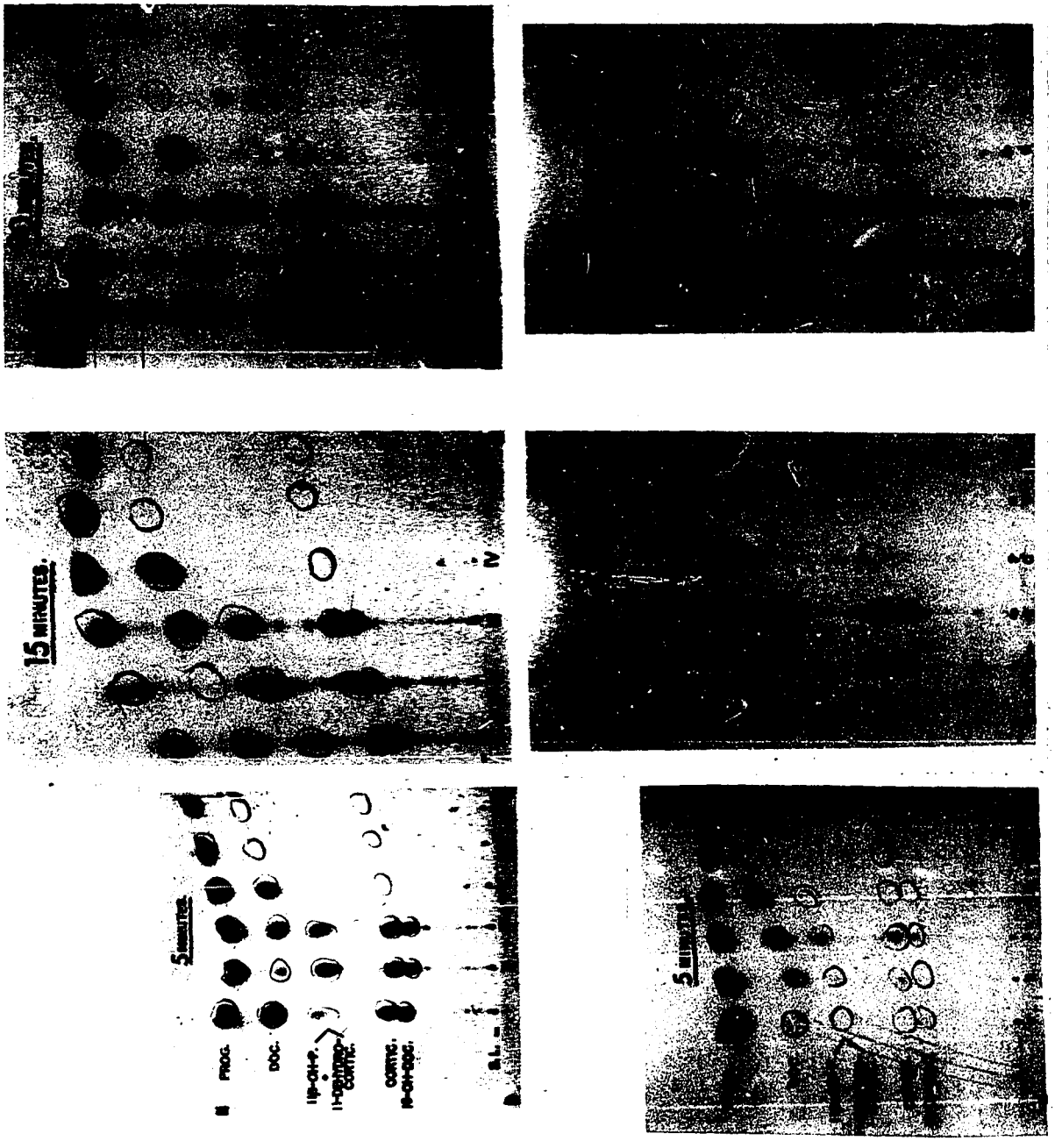

15 MINUTES.
5 MINUTES.
PROG.
DOC.
11β-OH-P.
11-DEHYDRO-CORTIC.
CORTIC.
N-OH-DOC.
S.M.

Figure 8

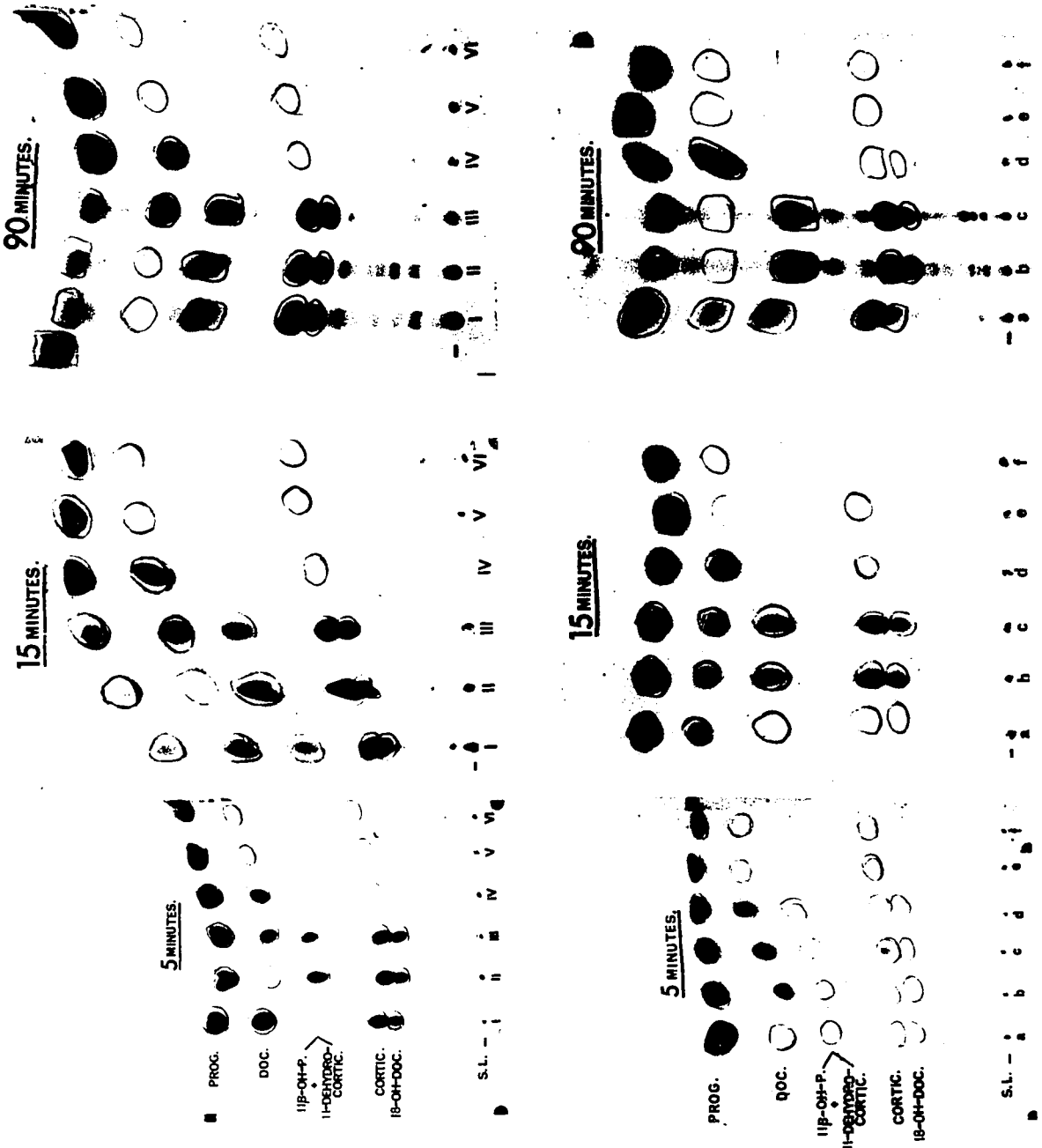
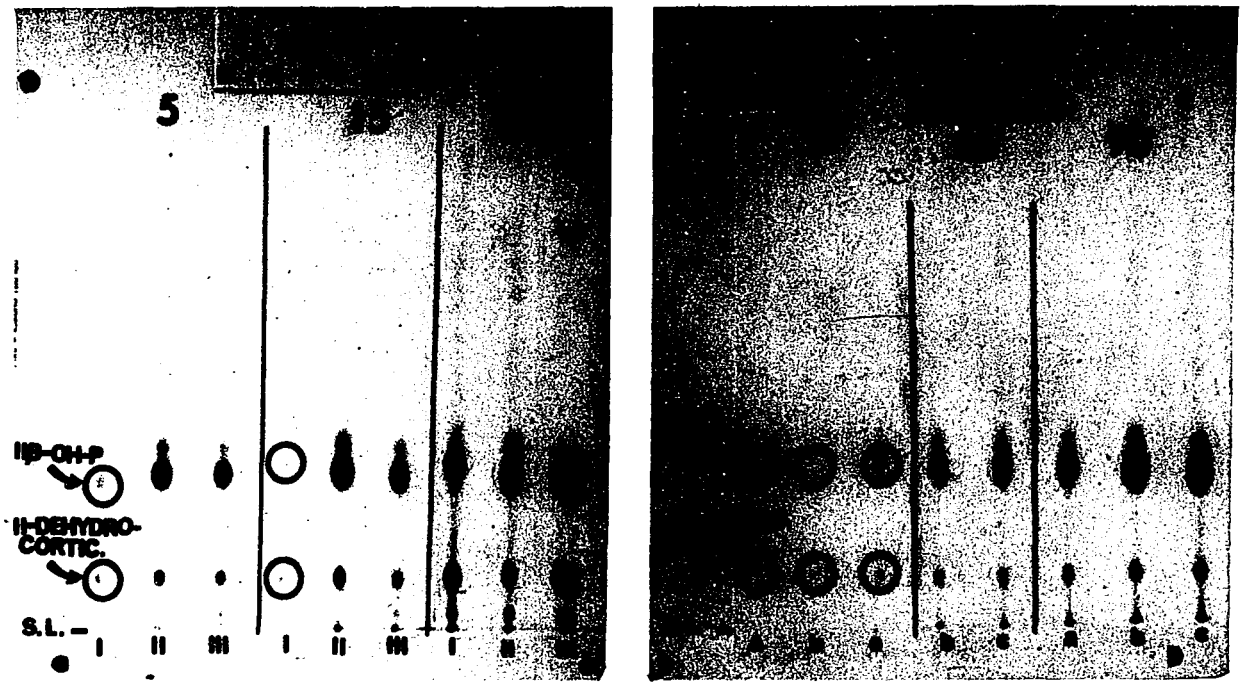

90 MINUTES.
15 MINUTES.
5 MINUTES.
PROG.
DOC.
11β-OH-P.
11-DEHYDRO-CORTIC.
CORTIC.
18-OH-DOC.
S.L.

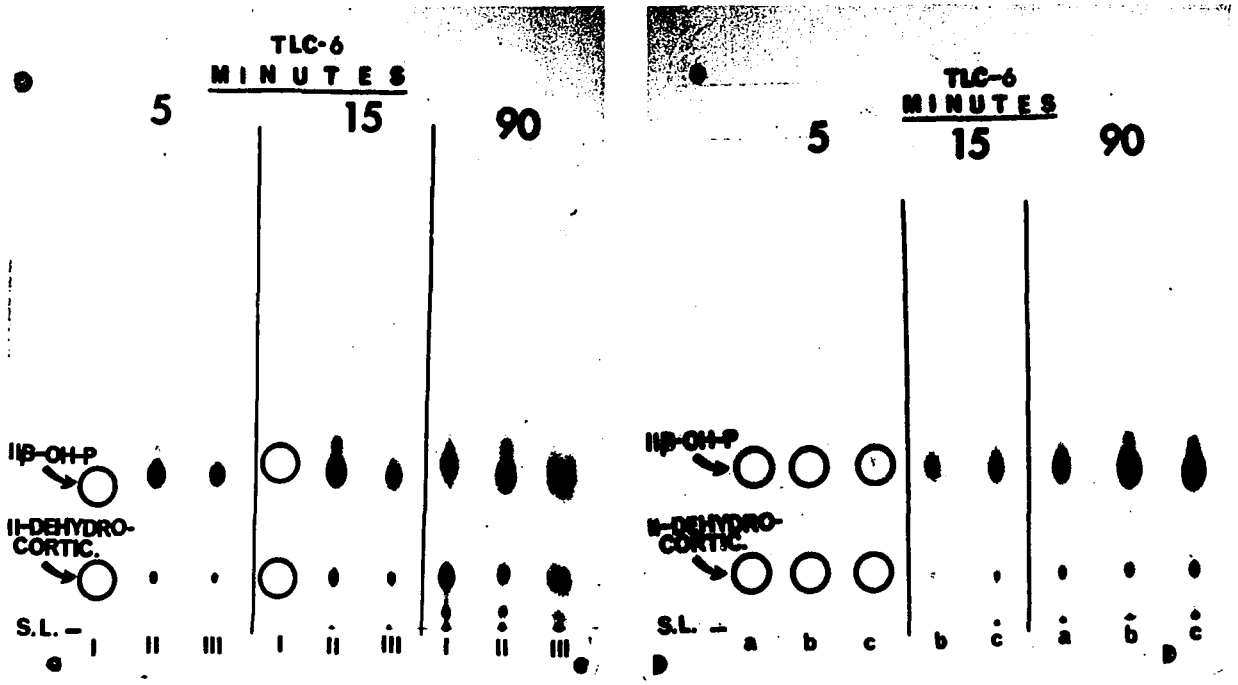
Figure 8



(i) (reduced scale) (ii)

Fig. 9. Radioautograms taken of chromatograms on TLC - 6 (Table I) (after TLC - 1 elution) showing the incorporation of 4-C^{14}-progesterone into 11β-hydroxyprogesterone (11β-OH-P) and 11-dehydrocorticosterone (11-dehydrocortic.) in dilute (ii) and concentrate (i) fasciculata homogenate of rat adrenals incubated for 5, 15 and 90 minutes under conditions shown in Table IX.

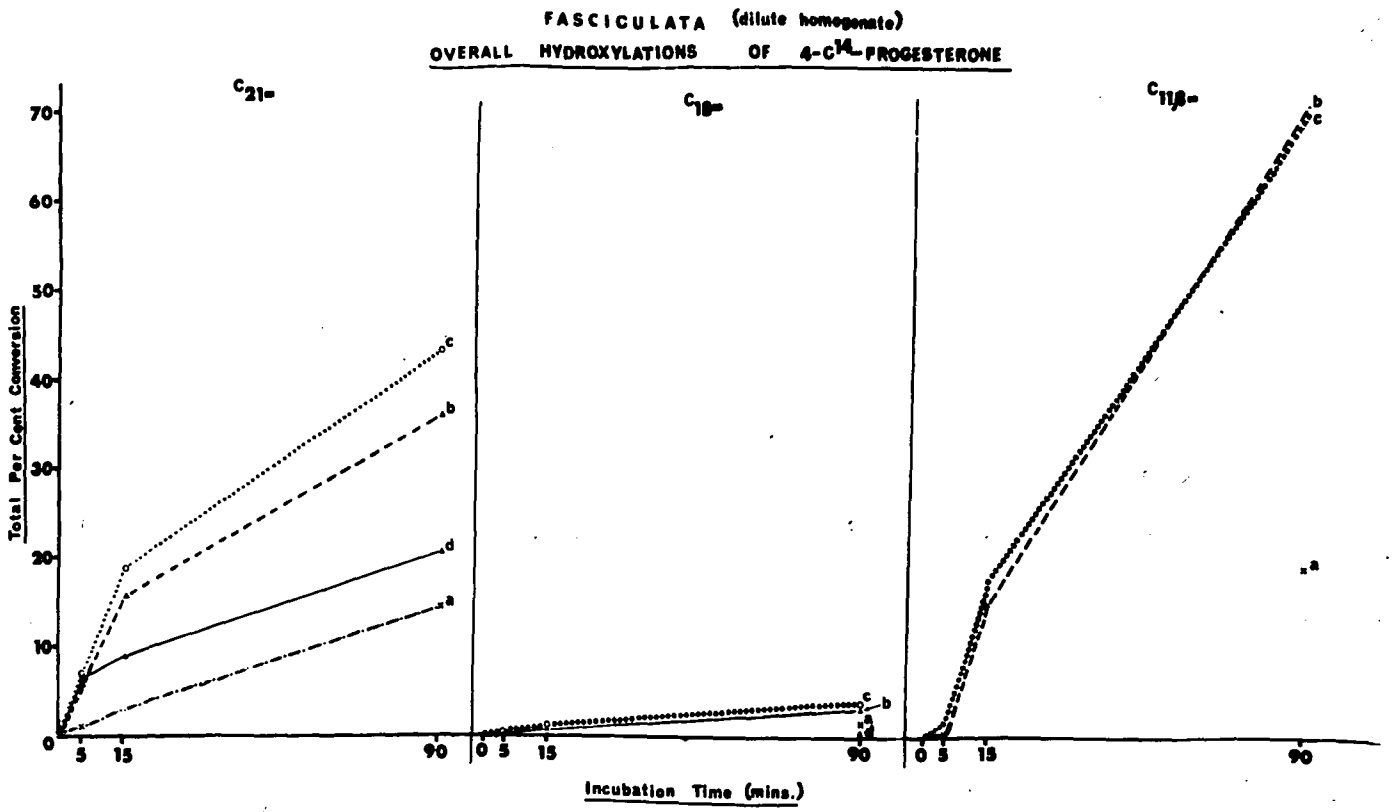
Legends to Fig. 9. I, II, III = concentrate fasciculata homogenate sample numbers; a, b, c = dilute fasciculata homogenate sample numbers; S. L. ___ = starting line (i.e., line of application on TLC chromatoplate); black circles = locations of the radioactive faint spots on original radioautograms to aid visualization.



(i) (reduced scale) (ii)

Fig. 9. Radioautograms taken of chromatograms on TLC - 6 (Table I) (after TLC - 1 elution) showing the incorporation of 4-C^{14}-progesterone into 11β-hydroxyprogesterone (11β-OH-P) and 11-dehydrocorticosterone (11-dehydrocortic.) in dilute (ii) and concentrate (i) fasciculata homogenate of rat adrenals incubated for 5, 15 and 90 minutes under conditions shown in Table IX.

Legends to Fig. 9. I, II, III = concentrate fasciculata homogenate sample numbers; a, b, c = dilute fasciculata homogenate sample numbers; S. L. ___ = starting line (i.e., line of application on TLC chromatoplate); black circles = locations of the radioactive faint spots on original radioautograms to aid visualization.



(i) (ii) (iii)

Fig. 10. Graphical presentation of the data of Table X showing the relative differences in the total 21, 18- and 11β-hydroxylation activities on 4-C^{14}-progesterone precursor (2,225,000 d.p.m. or 17.52 mμmoles per sample) in dilute fasciculata homogenate of rat adrenals incubated under conditions given in Table IX. The delay in 18- and 11β-, (but not 21-) hydroxylations between 0 - 5 minutes, and the differences in the rate of change of slope are their important differences to be noted.

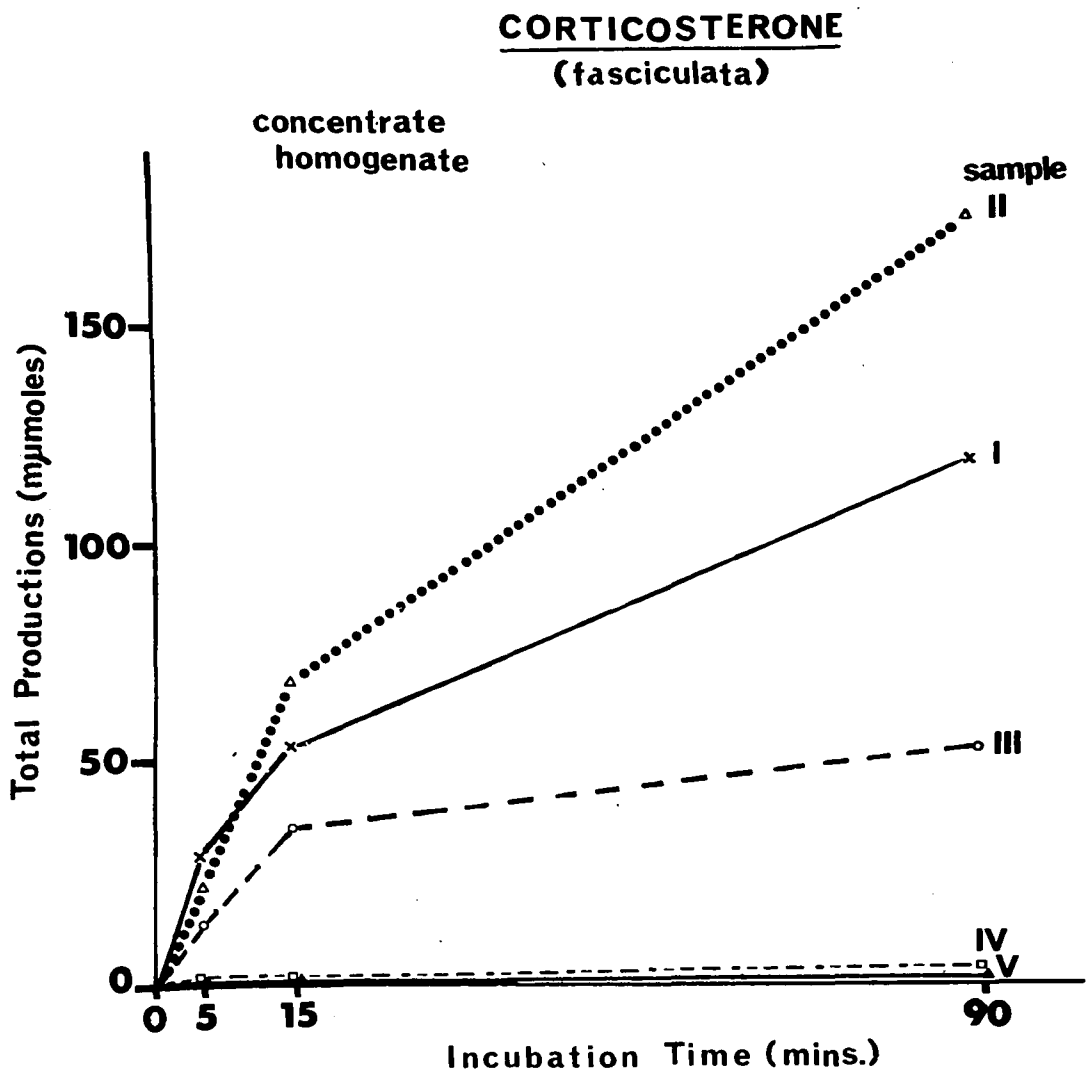


Fig. 11. Graphical presentation of data of Table XII showing the relative differences in the productions (total) of the major 11β-hydroxylated product of progesterone with respect to NADPH concentrations and time in concentrate fasciculata homogenate of rat adrenals incubated under conditions shown in Table IX. Values of samples IV and V (not shown in Table XII) were approximate and served only for comparison purposes.

Experiment III

The effect of gland integrity on adrenocorticoid hydroxylations in vitro, using 4-C^{14}-progesterone and 4-C^{14}-11-deoxycorticosterone precursors.

In an incubation of tissues with 4-C^{14}-precursors, it is generally assumed that the steroids in the incubation medium and at the site of synthesis are freely interchangeable (162). Consequently, the added 4-C^{14}-precursor mixes well with the endogenous precursor and the percent conversion is then an indication of the efficiency of the enzymatic system under the experimental conditions used.

As shown in Experiments I and II, in order to obtain more complete information on the in vitro adrenocorticoid biogenesis, it is necessary to know not only the percent conversion, but also to determine the endogenous production of the gland, and the dilution of the added precursor at the end of the incubation. In this experiment, the effect of the integrity of the rat adrenal on adrenocorticoid production in vitro, using 4-C^{14}-progesterone and 4-C^{14}-11-deoxycorticosterone as precursors, has been studied. Since it has been reported that the respiration of the whole intact adrenal was comparable to the sectioned adrenal (SAFFRAN et al. (284)), the valid comparison of the production by the intact whole rat adrenals and of the quartered rat adrenals was made.

The objective of the study was to elucidate information concerning:

(i) the precursor penetration as well as the steroidogenic capacity by the whole, intact gland compared to the quartered gland,

(ii) the amount of 4-C^{14} conversion product left in the gland after 2 hours of incubation, which was regarded negligible (since it has been reported that perfused gland released corticosteroid in the adrenal effluent almost as rapidly as it was formed, with no intracellular accumulation (23, 162)), and

(iii) the general assumption that there is a good homogeneity between intra- and extracellular compartments.

1. Experimental

Adrenals were obtained from 16 rats (210 - 250 gm. body weight). The 32 adrenals were cleaned from fat and distributed into four groups A, B, C and D of 8 glands each, in incubation beakers containing cold KRBG. The glands in A and B, or C and D, were from the same rat always. A, B, C and D each contained 4 right and 4 left glands, in order to minimize the effects of gland disparity. After the 1 hr. (2) preincubation at room temperature (22°C.) (2, 189), the glands were weighed. The weights of the tissues were: A 130 mg., B 115 mg., C 122 mg. and D 128 mg.. Only the glands in B and D were

quartered. The incubation lasted 2 hours at 37°C. in 4 ml. KRBG. There were no cofactors added but 3.1 mμmoles of 4-C^{14}-progesterone (specific activity 102,400 d.p.m./mμmole) in A and B, and 2.04 mμmoles of 4-C^{14}-11-deoxycorticosterone (specific activity 77,800 d.p.m./mμmole) in C and D.

At the end of the incubation, the supernatant of the incubation beakers were decanted after the termination of the reactions with 5 volumes of acetone. The glands and the beakers were rinsed with 1 ml. KRBG, and the rinsings were combined with the supernatant. This MEDIA A, B, C and D were divided into two known aliquots I and II. The glands collected in acetone were homogenized with 3 ml. distilled water in an all-glass power-driven homogenizer for 1 minute. The homogenate was divided into two known aliquots I and II. The two respective aliquots of the media and the glands were processed as described previously (Chapter IV (B) 3, 4 and Figure 6).

2. Results and Discussion

The results of this experiment are given in Tables XIV - XVII and Figure 12 at the end of the experiment. The radioautograms of the TLC - 1 chromatoplates (developed after 4 days' exposure) of the extracts of aliquot I are shown in Figure 12 (reduced scale).

A. General Considerations

It can be seen, in Fig. 12, that there were significant amounts of radioactivity incorporated mostly into 18-hydroxycorticosterone, aldosterone, 18-hydroxy,11-deoxycorticosterone and corticosterone. The relative incorporation of radioactivity into each of these compounds differed significantly between the whole and the quartered adrenals, and much higher values were found in the media than their respective tissues, as Fig. 12 suggested.

The percent conversions of either 4-C^{14}-progesterone or 4-C^{14}-11-deoxycorticosterone into the different compounds studied were tabulated in Table XIV. The fact that less 4-C^{14}-progesterone was used than 4-C^{14}-11-deoxycorticosterone in steroidogenesis was insignificant, since the total radioactivity of 4-C^{14}-11-deoxycorticosterone administered (15.9×10^4 d.p.m.) was only half as much as the radioactivity of 4-C^{14}-progesterone (31.8×10^4 d.p.m.) added as steroid precursors. From Table XIV, the following can be noted.

B. Percent Conversions

(a) 4-C^{14}-progesterone versus 4-C^{14}-11-deoxycorticosterone. The percent conversion into aldosterone and corticosterone did not differ appreciably regardless of the two precursors used, when whole glands were compared with the whole glands, or quartered with quartered glands. The

exception to this was the 11-deoxy-compound.

(b) Tissue versus medium. The percent conversion of the labeled precursors into the different products was generally higher in the media than in the tissues, whether for the whole or quartered glands. Nevertheless, the tissues of the whole glands contained up to about 33 - 50% of the 4-C^{14}-corticosterone and 50% of aldosterone found in their respective media. Approximately 14 - 33% of corticosterone and 14 - 33% of aldosterone of the quartered-gland media were found in the quartered-gland tissues (Table XIV).

(c) Whole gland versus quartered gland. The percent conversions of the 4-C^{14}-precursors to aldosterone and 18-hydroxycorticosterone were much more pronounced in the whole-gland tissues, being three to four times higher than in the quartered-gland tissues. On the other hand, the quartered glands had a two-or three-time higher percent conversion to corticosterone and 18-hydroxy,11-deoxycorticosterone than the whole glands, whether in the tissues or in the media (Table XIV).

C. Specific Activities

(a) The specific activities (d.p.m./mμmole) of aldosterone, corticosterone, progesterone and 11-deoxycorticosterone are shown in Table XVI. The data indicated that the specific activities of all the steroids obtained were much higher in

the media than in the tissues, an observation which strongly supports the view that at least two different pools of steroids exist. It can be postulated that the quantity of steroid in the media represents the sum of the amount of steroid released by the tissue cells into the media plus the quantity synthesized in the medium itself. This "exogenous" quantity could be produced from the labeled precursors added through the action of the enzymes leaked into the medium (i.e., extracellular enzymes) (3, 2) and by some damaged cells with abnormally high membrane permeabilities (285). The steroids produced in this manner would have a relatively high radioactivity despite their minute quantities produced. This plus the probably limited mixing with the endogenous steroid pool(s) would explain, at least in part, the relatively higher specific activities of steroids obtained from the media.

(b) The specific activities of 4-C^{14}-progesterone were significantly higher than its various conversion products, both in the tissues and in the media. Thus, it seems probable that either the formation of endogenous progesterone was very small, and the precursor other than progesterone (158 - 160, 147) might have given rise to aldosterone, corticosterone and 11-deoxycorticosterone, or that the added 4-C^{14}-progesterone were not well mixed with the in situ progesterone, which was converted as soon as it was formed and without being released from the intracellular compartment.

(c) In the tissues, the specific activities of DOC derived from 4-C^{14}-DOC were slightly smaller than or equal to those of corticosterone. This could indicate that DOC was an intermediate as well as a normal precursor of the steroids studied.

D. Total Productions

(a) The total productions (mμmoles) per 8 glands of the different steroids studied were calculated according to the method described previously (Chapter IV (B) 6 and Figure 6) and tabulated in Table XVII, which indicated that the steroids obtained were present in very significant concentrations in the quartered or whole-gland tissues.

(b) The total quantity of aldosterone produced by the whole glands (14.8 mμmoles), calculated by adding the production in the tissue and that in the respective medium (Table XVII, column (3)), was almost twice that of the quartered glands (7.9 - 8.3 mμmoles).

(c) Despite the quantity of corticosterone remaining in the tissue of whole and quartered glands was almost the same, the quantity of corticosterone released into the media of the quartered aldns was twice that of the whole glands.

(c) Similar to aldosterone (Table XVII), the 18-hydroxy-corticosterone content (Table XV) of whole-gland tissues

was four to five times that of the quartered-gland tissues. On the other hand, the quartered-adrenal tissues showed a two- to three-time higher production of 18-hydroxy,11-deoxycorticosterone than the whole-gland tissues. It is already known that, in the fasciculata tissue, the main products formed were 18-hydroxy,11-deoxycorticosterone and corticosterone (Experiments I and II) and there was no 18-hydroxycorticosterone. In the glomerulosa tissue, however, there was 18-hydroxycorticosterone and aldosterone besides corticosterone, all in small quantities. The difference between whole and quartered adrenals could be due to the fact that the corticosterone and 18-hydroxy,11-deoxycorticosterone produced in the fasciculata of the whole gland had to pass through the glomerulosa tissue to be released in the media and that the enzymatic system of the glomerulosa, presumably not saturated with substrates, converted them to form more aldosterone and 18-hydroxycorticosterone. The overall result would be more aldosterone and 18-hydroxycorticosterone, but less corticosterone in the whole-gland tissues. Thus, the steroids secreted into the medium by the whole-gland tissues might represent integrated hydroxylation activities of the two zones of the adrenal cortex. Hence, the effect of the integrity of the adrenals on steroidogenesis in vitro could be anticipated.

(d) It is interesting to note that the quantity of DOC and progesterone was higher in the tissues than in the media. This might be due to the fact that the tissue cells had higher retention capacities of steroids which served as potential biosynthetic intermediates.

It can be noted, in Table XVII, that the exogenous productions (i.e., the productions originated from the labeled precursors added) were very small and these precursors served ideally as tracers without contributing much to the total steroid productions.

E. Concluding Remarks

(a) The 4-C^{14}-precursors added were able to pass through the cell membrane and even through the capsule of the whole gland. Although in the quartered glands their utilization was better, the whole glands retained more of the conversion products formed, especially the aldosterone and 18-hydroxycorticosterone. The whole, intact glands incubated were able to produce steroids and to hydroxylate labeled progesterone. As already mentioned, there was a possibility of the recirculation of the steroids produced by the internal zone of the gland (zone fasciculata) into the zona glomerulosa, especially in the whole glands.

(b) The consideration of the amounts of 4-C^{14} conversion

products (mμmoles) left in the tissues of the whole and quartered glands after the incubation could not be considered to be negligible.

(c) Under the experimental conditions described in this Thesis, the incubation of tissue slices or whole glands cannot be considered as a homogeneous system, since the application of a labeled steroid precursor in the system demonstrated the different specific activities of the steroids in the media and in the tissues. Accordingly, at least two pools of steroids exist and they do not seem to mix well. As shown in Table XVII, the amounts of 4-C^{14} conversion products (exogenous production) left in the gland after 2 hours of incubation was dependent on the conversion product concerned as well as the integrity of the gland. The existence of at least two pools of steroids in the in vitro system pointed to the great importance of the need of a thorough study of the incubation media in such investigations, besides rendering the general assumption that there is a good homogeneity between intra- and extracellular compartments questionable.

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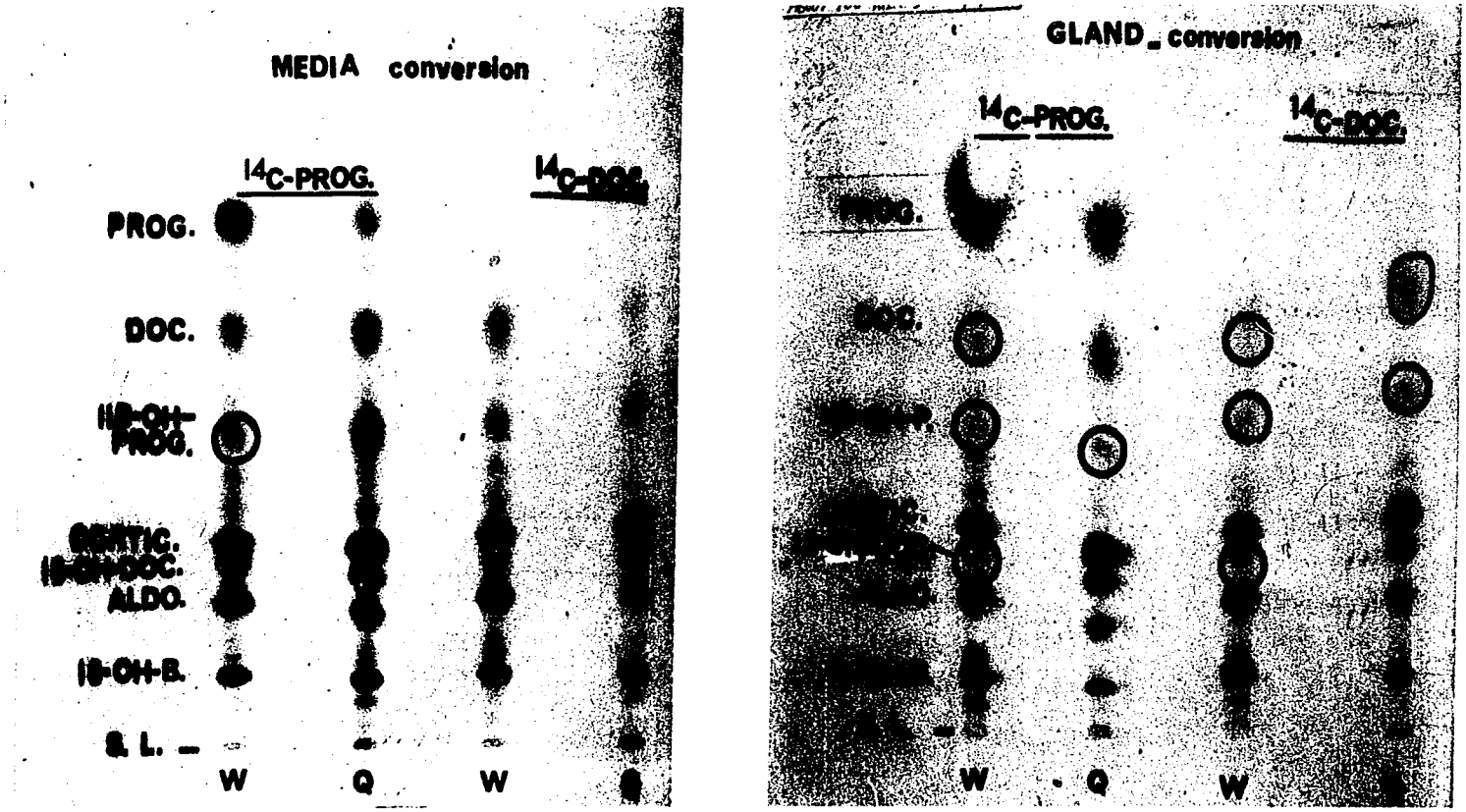
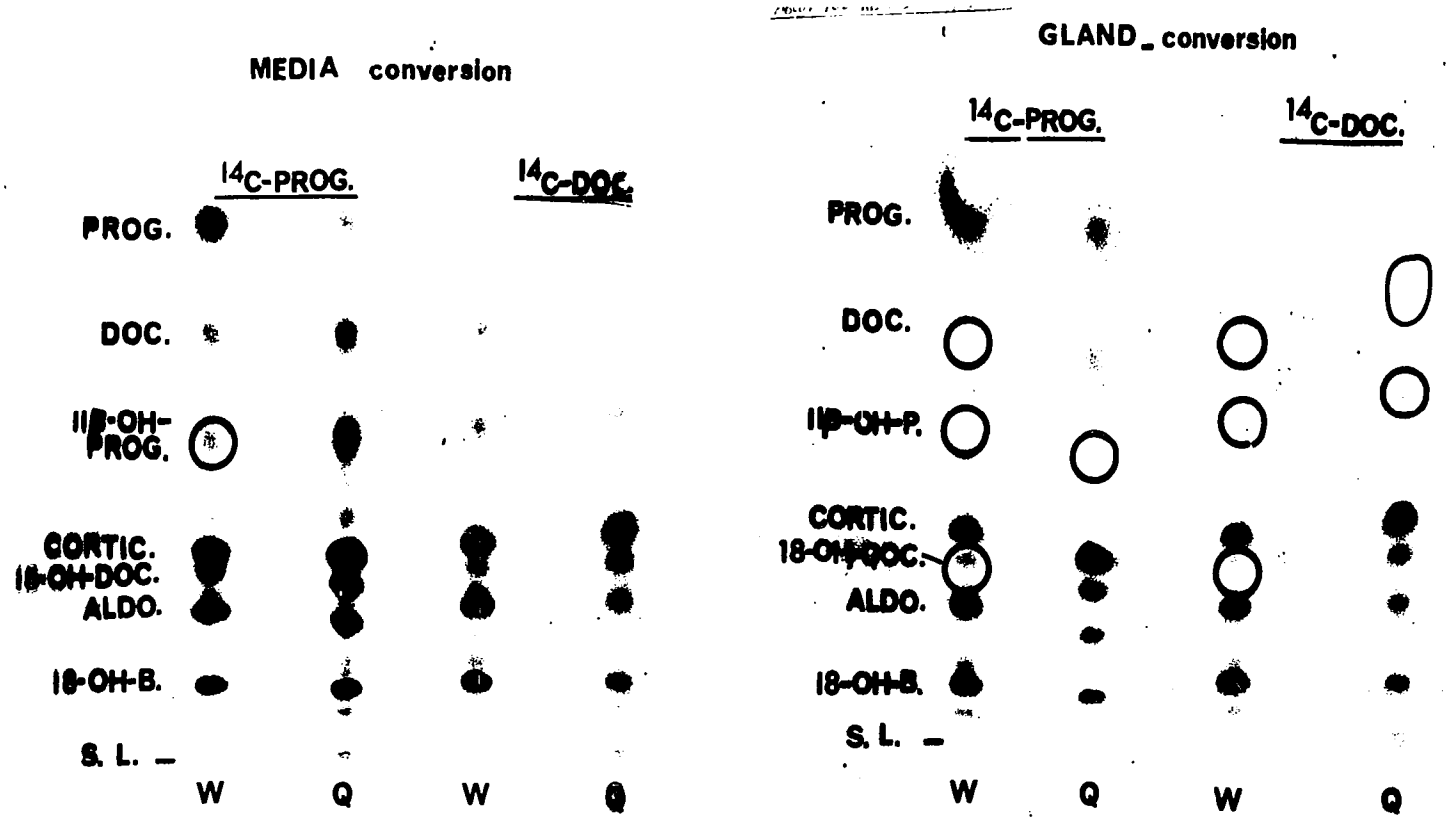


(i) (reduced scale) (ii)

Fig. 12. Radioautograms taken of TLC chromatograms on TLC - 1 (Table I) (developed after 100 hrs. of exposure) showing the incorporation of 4-C^{14}-progesterone and 4-C^{14}-11-deoxycorticosterone into different steroids when incubated with whole (W) intact and quartered (Q) rat adrenals under the conditions described in Experimental.

Legends to Fig. 12. S.L. __ = starting line (i.e., line of application on the chromatoplate); black circle = location of faint radioactive spot on the radioautogram to facilitate visualization.



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Legends to Fig. 12. S.L. __ = starting line (i.e., line of application on the chromatoplate); black circle = location of faint radioactive spot on the radioautogram to facilitate visualization.

TABLE XIV

PERCENT CONVERSION OF 4-C^{14}-PROGESTERONE AND 4-C^{14}-DOC TO DIFFERENT 21-, 18- and 11β-HYDROXYLATED STEROIDS BY WHOLE (W) AND QUARTERED (Q) GLANDS AND THEIR RESPECTIVE MEDIA **

Conversion Product	4-C^{14}- Precursor	(i) Tissue		(ii) Media		Total* = (i) + (ii)	
		W	Q	W	Q	W	Q
Aldosterone	Prog.	5.4	1.9	8.9	6.9	14.3	8.8
	DOC	4.5	1.1	10	7.4	14.5	8.5
Corticosterone	Prog.	3.1	9	7.3	26.8	10.4	35.8
	DOC	3.2	8.2	9.8	22.8	13	31
11-deoxycorticosterone	Prog.	1	3.1	1.4	2.3	2.4	5.4
	DOC	0.7	1.9	2.3	1.3	3	3.2
Progesterone	Prog.	15.1	6.9	2.7	0.9	17.8	7.8

** Incubation conditions: 37°C. under 95% O_2 - 5% CO_2 for 2 hrs. in KRBG,

4-C^{14}-progesterone: 3.1 mμmoles added, specific activity 102,400 d.p.m./mμmole

4-C^{14}-DOC : 2.04 " " , specific activity 77,800 d.p.m./mμmole

* calculated W in Total by adding W of (i) to W of (ii)
Q " " " " Q " " " Q " "

TABLE XV

PERCENT CONVERSION AND PRODUCTIONS (mμmoles) OF 18-OH-B AND 18-OH-DOC IN TISSUES AND MEDIA OF WHOLE (W) AND QUARTERED (Q) GLANDS

$4\text{-}C^{14}$-Precursor	18-OH-B								18-OH-DOC							
	Gland				Media				Gland				Media			
	P		DOC		P		DOC		P		DOC		P		DOC	
	W	Q	W	Q	W	Q	W	Q	W	Q	W	Q	W	Q	W	Q
* Percent Conversion	4.0	1.0	4.2	1.3	2.4	4.1	3.9	1.8	0.6	1.7	0.9	2.4	2.4	7.0	1.1	2.5
** Total Production	14.9	3.0	15.4	4.1	2.8	10.5	5.6	3.6	2.9	9.0	4.6	10.1	4.2	9.8	1.8	5.7

Incubation conditions are given in Table IX (p. 151)

* calculated after second chromatography (TLC - 2), allowing for a 40% recovery

** calculated from the iso-nicotinic acid hydrazide (INH) determinations, result of per 8 glands

TABLE XVI

SPECIFIC ACTIVITIES (d.p.m./mμmole) OF DIFFERENT STEROIDS IN THE INCUBATION MEDIA AND TISSUES OF WHOLE (W) AND QUARTERED (Q) GLANDS

Steroid Products	4-C^{14}- Precursor	Tissues		Media		Tissues + Media *	
		W	Q	W	Q	W	Q
Aldosterone	Prog.	1,940	2,180	4,730	4,230	3,070	3,520
	DOC	1,220	1,410	1,790	1,640	1,560	1,600
Corticosterone	Prog.	1,330	3,650	3,840	5,610	2,460	4,910
	DOC	690	1,700	2,190	2,730	1,430	2,360
11-deoxycorticosterone	Prog.	1,270	3,750	7,360	12,900	2,410	5,360
	DOC	540	1,280	5,800	4,880	1,800	1,820
Progesterone	Prog.	32,400	18,600	86,000	37,400	-	-

Incubation conditions as shown in Table XIV

Method of calculation of specific activities as shown in Chapter IV (B) 6

* calculated by the expression: (X.m + Y.n) / (m + n), where X, Y were specific activities of the steroid in tissue and its respective medium respectively; m, n were the total quantities of the steroid (in mμmoles) in the tissue and medium respectively

TABLE XVII

PRODUCTIONS (mμmoles/8 glands) OF DIFFERENT STEROIDS IN THE INCUBATION MEDIA AND TISSUES OF WHOLE (W) AND QUARTERED (Q) GLANDS

Steroid Produced	4-C^{14}- Precursor	(1) Tissues				(2) Media				(3) Tissues + Media			
		W		Q		W		Q		W		Q	
		Ex P	T P	Ex P	T P	Ex P	T P	Ex P	T P	Ex P	T P	Ex P	T P
Aldosterone	Prog.	0.17	8.8	0.06	2.8	0.28	6.0	0.21	5.1	0.45	14.8	0.27	7.9
	DOC	0.09	5.9	0.02	1.2	0.20	8.9	0.15	7.1	0.29	14.8	0.17	8.3
Corticosterone	Prog.	0.10	7.5	0.28	7.8	0.23	6.1	0.83	15.2	0.33	13.6	1.11	23.0
	DOC	0.07	7.3	0.17	7.6	0.20	7.1	0.47	13.3	0.27	14.4	0.64	20.9
11-deoxycorticosterone	Prog.	0.03	2.6	0.10	2.6	0.04	0.6	0.07	0.6	0.07	3.2	0.17	3.2
	DOC	0.01	2.0	0.04	2.3	0.05	0.6	0.03	0.4	0.06	2.6	0.07	2.7
Progesterone	Prog.	0.47	1.5	0.21	1.2	0.08	0.03	0.03	0.05	0.55	1.53	0.24	1.25

(3) obtained by adding the production in (1) to its corresponding production in (2)

Incubation conditions as shown in Table XIV

Legends: Ex P = Exogenous Production, T P = Total Production

Experiment IV

Studies on the hydroxylation of 11-deoxycorticosterone by the mitochondrial fractions of the cells of zona glomerulosa and of zona fasciculata.

As shown in Experiment I, there are differences in the 11β- and 18-hydroxylase systems of the zona glomerulosa and fasciculata. Accordingly, this experiment was designed to investigate the specificity of the 11β- and 18-hydroxylating systems of the glomerulosa and fasciculata of the rat adrenal cortex. It has been reported (185, 186) that the fasciculata tissue slices incubated gave rise to 18-hydroxy,11-deoxycorticosterone and corticosterone, with very little 18-hydroxycorticosterone and no aldosterone, while the glomerulosa counterpart yielded corticosterone, aldosterone and 18-hydroxycorticosterone but very small amount of 18-hydroxy,11-deoxycorticosterone. The effect of substrate concentrations on the 11β- and 18-hydroxylating activity of glomerulosa and fasciculata mitochondrial fractions was investigated with DOC as substrate.

1. Experimental

42 rat adrenals dissected free from surrounding fat were separated into zona glomerulosa and fasciculata-reticularis (5). After preincubation at 37°C. for 30 minutes, 232 mg. glomerulosa tissue and 436 mg. of fasciculata tissue were homogenized in cold 0.44 M sucrose solution with a pestle

homogenizer at low speed for 1 minute (Chapter IV (B) e). The homogenates were centrifuged at 750 g for 10 minutes in a refrigerated ultracentrifuge. The supernatant was decanted and centrifuged at 13,300 g for 10 minutes. The mitochondrial fractions of glomerulosa and fasciculata so obtained were carefully re-homogenized in 10 ml. sucrose separately.

To 16 vials each containing 1 ml. KRBG, 10 µmoles sodium malate, 33.5 µmoles G-6-P, 4.8 µmoles NADP and 5 units of G-6-P-dehydrogenase, 1 ml. of fasciculata mitochondria solution was added to eight of them, and 1 ml. of glomerulosa mitochondria solution to the other eight. In addition, these vials contained DOC and 4-C^{14}-DOC (specific activity 77,900 d.p.m./mµmole) of known amounts, the total of which were shown in Table XVIII. Each vial contained the mitochondrial fraction corresponding to 4 glands. The protein determination gave a concentration of 948 ug. protein corresponding to 22 mg. glomerulosa tissue of 4 glands, and 9140 ug. protein corresponding to 41.2 mg. fasciculata tissue of 4 glands. The total final volume of the contents was 4 ml./vial.

The incubation vials were incubated in a Dubnoff incubator at 37°C. for 15 minutes. After the addition of known amounts of tritiated DOC, aldosterone and corticosterone, they were extracted and processed to obtain the percent conversion

values. Since there was no means of recovery for 18-hydroxycorticosterone and 18-hydroxy,11-deoxycorticosterone, they were calculated after the periodic acid oxidation and TLC - 2 (Table I),allowing for a 40% recovery.

2. General Considerations

The glomerulosa and fasciculata mitochondrial fractions incubated in each vial corresponded to 4 glands. The purpose of this consideration was to make a comparison of the different hydroxylase activities of the two tissues on the gland basis. From the results obtained for the protein determination, it can be seen that the quantity of protein/gland (238 µg.) of the glomerulosa was 10 times smaller than the protein/gland (2290 µg.) of the fasciculata tissue.

The range of substrate concentrations used in this experiment was very wide, from 1.2 mµmoles to 760.9 mµmoles of DOC, since there has yet been no evidence that the enzymes concerned in the conversion of corticosterone to aldosterone are easily saturated with low concentrations of substrates (151, 185). 4-C^{14}-DOC was also used since it was easier to trace the different conversion products, to measure the percent conversions and hence the mµmoles produced.

Although the 4-C^{14}-DOC had been thoroughly purified just before use, a control sample consisting of the same batch

of 4-C^{14}-DOC, cofactors, etc., as other samples but with no mitochondria solution added, when incubated and extracted showed on the TLC chromatoplate an impurity. This "impurity" was running between corticosterone and aldosterone in TLC - 1 and could be seen in samples 1 to 8 (glomerulosa preparation) but not in the rest (fasciculata preparation).

3. Results and Discussions

The results of this study are presented in Table XVIII and Figures 13 - 15 at the end of this experiment.

It can be seen that the enzymatic systems of the fasciculata (samples 9 - 16) were much more active than those of the glomerulosa. The DOC substrate added was always in excess, even at the smaller substrate concentration, in the incubation with glomerulosa mitochondrial fraction (samples 1 - 8), whereas in the incubation with the fasciculata mitochondrial fraction there was a small excess only for the two highest concentrations (samples 15, 16).

In glomerulosa, the main steroids from the substrate DOC were corticosterone, 18-hydroxy,11-deoxycorticosterone, aldosterone and 18-hydroxycorticosterone. In fasciculata, there was corticosterone, 18-hydroxy,11-deoxycorticosterone, but very small amounts of 18-hydroxycorticosterone and no aldosterone. Such findings were reminiscent of the previous

results of other investigators (185,186) working with slices.

The aldosterone, although present on the radioautograms of TLC - 1 (not shown) in the glomerulosa samples, was not measured due to some difficulty encountered during the processing.

A. Glomerulosa

With reference to Table XVIII and Figures 13 - 15, it is conceivable that corticosterone was produced in increasing amounts with an increase in the substrate, but the production plateaued at 13.5 mμmoles of DOC. At higher concentrations of DOC, there was a substrate inhibition.

For 18-hydroxycorticosterone, the production from DOC was small and increased to reach a plateau at a lower concentration of substrate, 6.0 mμmoles. Thereafter there was a substrate inhibition. The quantity of 18-hydroxy,11-deoxycorticosterone formed was higher than that of 18-hydroxycorticosterone, being increased steadily with the increase in substrate to reach a plateau at 13.5 mμmoles of substrate. A substrate inhibition was also shown at higher substrate concentration.

B. Fasciculata

It was only in the last sample (760.9 mμmoles DOC)

that the substrate was in excess. Corticosterone production increased with increasing concentrations of substrate. 18-hydroxycorticosterone was produced in very small quantities in fasciculata at the lower substrate concentrations, but demonstrated a steady increase until the highest substrate concentration (760.9 mμmoles DOC) was reached. It was inhibited by this substrate concentration.

C. Glomerulosa versus Fasciculata

For both the glomerulosa and fasciculata mitochondrial preparations, the 11β-hydroxylation of DOC was always two to three times greater than the corresponding 18-hydroxylation.

The difference between the two zones of the adrenal cortex seemed to be that at the lower concentrations of substrate (DOC) more 18-hydroxycorticosterone and less 18-hydroxy,11-deoxycorticosterone were formed by the glomerulosa from the same quantity of substrate added. There was too a much higher enzyme activity (11β- and 18-hydroxylases) in the fasciculata, since the fasciculata mitichondrial fraction was able to produce 195 μmoles of corticosterone out of 760.9 mμmoles of substrate without being completely saturated. However, the glomerulosa mitochondria were saturated with only 13 mμmoles of substrate, producing 1.24 mμmoles of corticosterone.

The above considerations are in agreement with the fact previously underlined, i.e., the quantity of protein/gland, which can, in a crude way, indicate that the enzymatic activity, is 10 times smaller in glomerulosa than in the fasciculata. Consequently, as a result of low enzymatic activities, there was a saturation with a relatively low substrate concentration of DOC in the glomerulosa but not in the fasciculata. It is difficult to ascertain if at concentrations much higher than those tested in this experiment the same phenomenon (i.e., inhibition) will not occur with the fasciculata mitochondria.

With the available data of Table XVIII, Lineweaver-Burk reciprocal plots for the 11β- and 18-hydroxylations are presented in Figures 13 - 15. In these plots, the values obtained at the lower substrate concentrations for glomerulosa and at the higher substrate concentrations for the fasciculata were taken. From these graphical approaches, the Michaelis constants (K_m) of the 11β- and 18-hydroxylases of the glomerulosa and fasciculata mitochondrial fractions were as follows:

mitochondrial fraction	hydroxylase K_m	
	11β-	18-
glomerulosa	1.6×10^{-8} M	2×10^{-8} M
fasciculata	0.7×10^{-6} M	0.3×10^{-6} M

The author is aware that these values presented above can be subject to criticism on some points. However, under the experimental conditions described in this Thesis, they reflected the much higher substrate affinity of the 11β- and

the 18-hydroxylases in the glomerulosa compared to those of the fasciculata.

D. Concluding Remarks

Definite conclusions cannot be drawn from these data as to the specificity of these enzyme systems in the glomerulosa and fasciculata mitochondrial preparations. They have to await for further clarification. What can be remarked about them is that, owing to the smaller 11β- and 18-hydroxylase activities in the glomerulosa, these enzyme systems are much more susceptible to substrate (DOC) inhibition than those of the fasciculata.

Despite of its small capacity, the formation of 18-hydroxycorticosterone occurred more readily in the glomerulosa mitochondria. In other words, 18-hydroxylation of corticosterone or 11β-hydroxylation of 18-hydroxy,11-deoxycorticosterone was more difficult in fasciculata compared to the glomerulosa counterpart. The data obtained in this experiment could not resolve the question whether the 18-hydroxylation of corticosterone or the 11β-hydroxylation of 18-hydroxy,11-deoxycorticosterone that was impaired in the fasciculata tissue. Accordingly, the following experiment was designed to investigate the 18-hydroxylation of corticosterone in the mitochondrial fractions of these two zones of the adrenal cortex.

TABLE XVIII

HYDROXYLATION PRODUCTS (mμmoles) OF DOC BY GLOMERULOSA AND FASCICULATA MITOCHONDRIAL FRACTIONS (15 min. incubation at 37°C.)

Sample Z.G. Z.F.	Total Substrate, S, per 4 ml, (DOC + 4-C^{14}-DOC) mμmoles	DOC Recovered	18-OH DOC	Corticosterone	18-OH-B
1	1.2	0.31	0.09	0.34	0.03
2	2.4	0.76	0.20	0.64	0.06
3	6.0	3.20	0.40	1.04	0.07
4	13.5	10.80	0.49	1.39	0.06
5	43.8	35.70	0.57	1.10	0.04
6	156.7	155.10	0.25	1.25	-
7	307.0	303.90	0.37	1.23	-
8	760.9	753.30	0.30	0.76	-
9	1.2	0.007	0.16	0.58	0.01
10	2.4	0.14	0.36	1.05	0.02
11	6.0	0.02	0.70	2.50	0.06
12	13.5	0.11	2.40	5.50	0.09
13	43.8	0.22	8.20	18.30	0.25
14	156.7	0.63	30.90	68.50	0.55
15	307.0	4.90	75.40	127.40	0.74
16	760.9	249.60	110.30	220.70	-

Details of incubation conditions are given in Experimental (pages 155, 156).

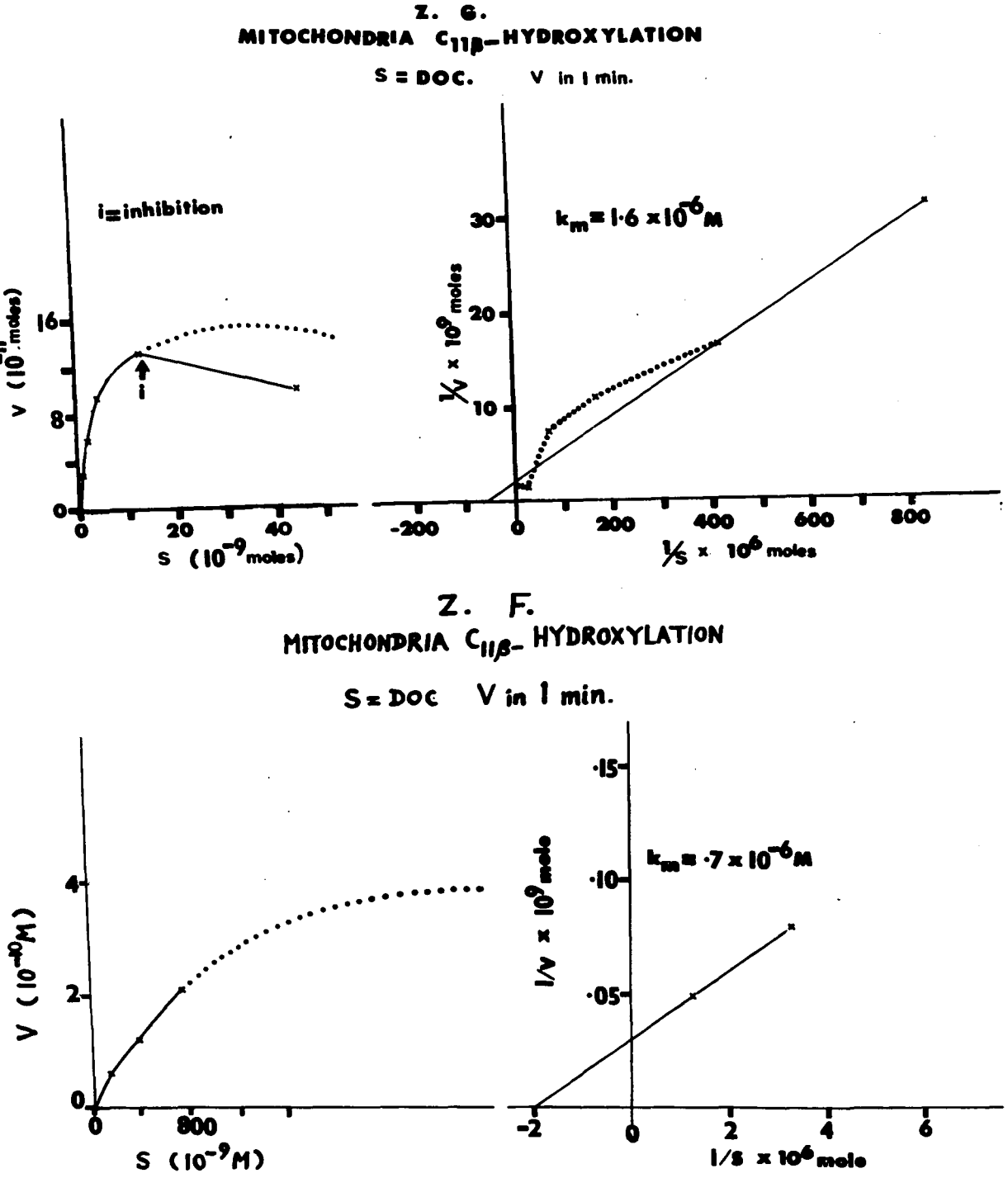


Fig. 13. Graphical presentation of the 11β-hydroxylation of 11-deoxycorticosterone (formation of corticosterone) in zona glomerulosa (Z.G.) and zona fasciculata (Z.F.) mitochondrial fractions incubated under conditions described in Experimental. Dotted curves were extrapolations. K_m = Michaelis constant, S = substrate concentration (or substrate), V = velocity of reaction (mμmoles or smaller units/minute).

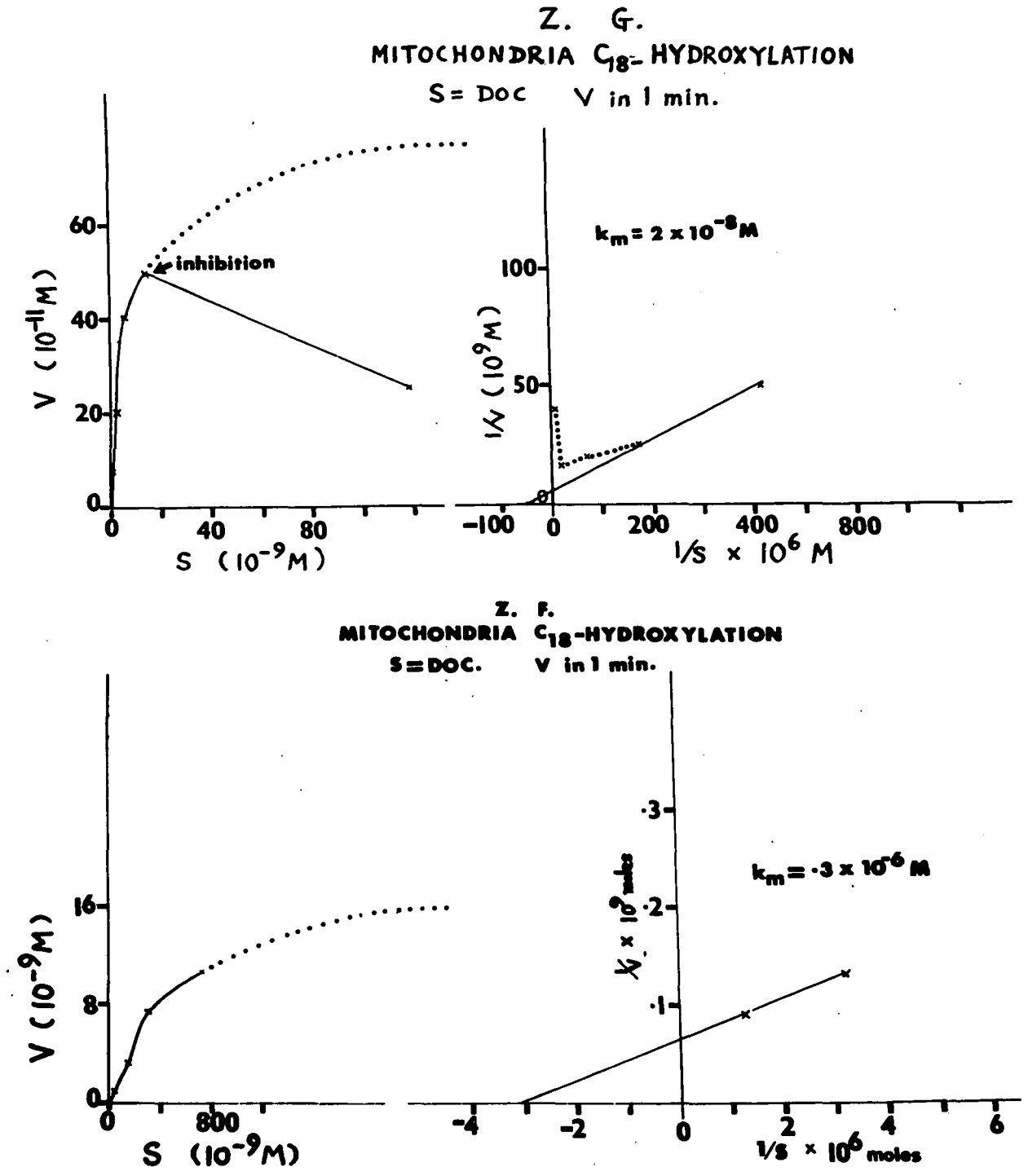


Fig. 14. Graphical presentation of the 18-hydroxylation of 11-deoxycorticosterone (formation of 18-hydroxy,11-deoxycorticosterone) in zona glomerulosa (Z.G.) and zona fasciculata (Z.F.) mitochondrial fractions under the conditions described in Experimental. Dotted curves were extrapolations. K_m = Michaelis constant, S = substrate concentration (or substrate), V = velocity of reaction (mμmoles or smaller units/minute).

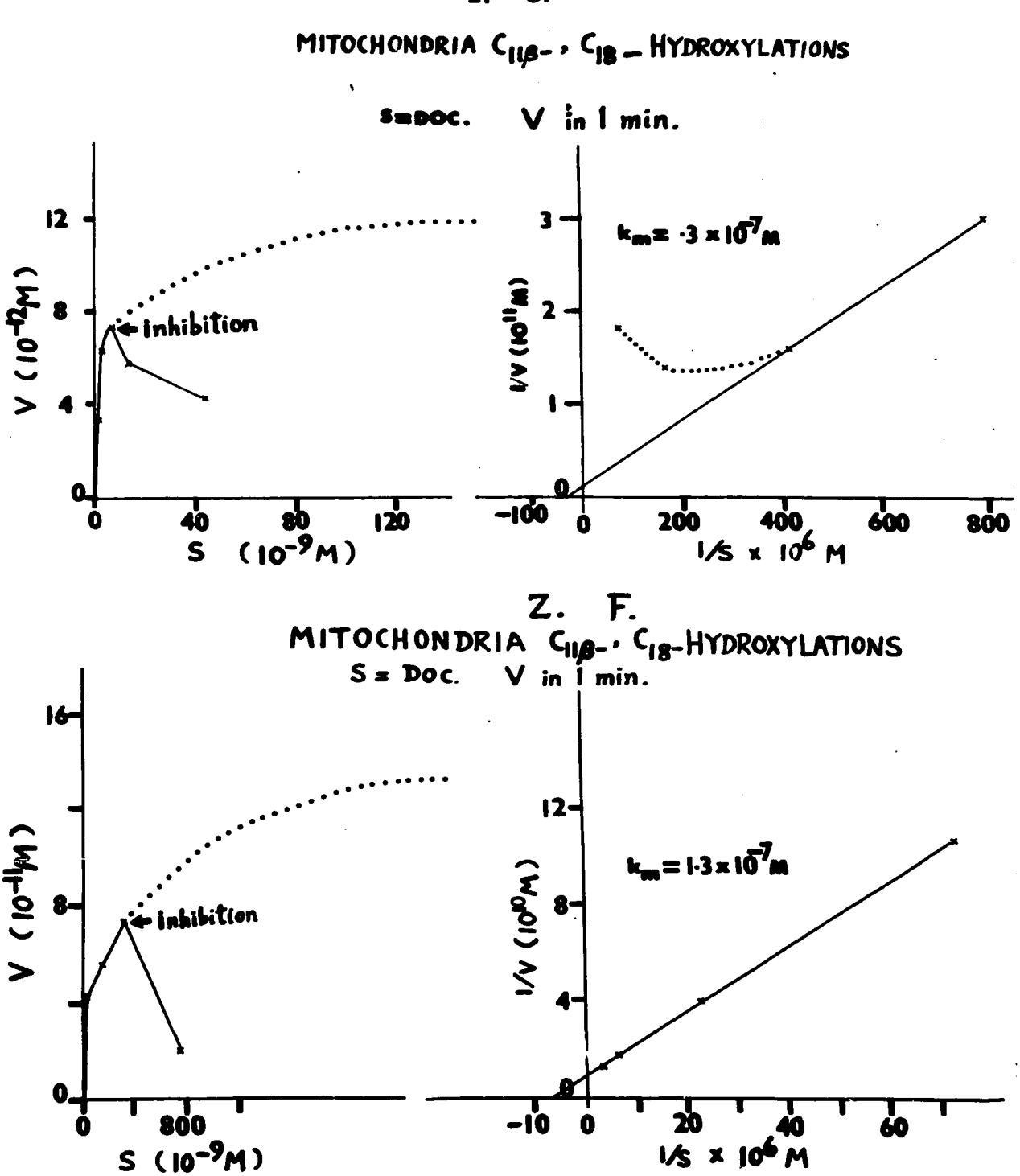


Fig. 15. Graphical presentation of the 11β- and 18-hydroxylations of 11-deoxycorticosterone (formation of 18-hydroxycorticosterone) in zona glomerulosa (Z.G.) and zona fasciculata (Z.F.) mitochondrial fractions incubated under conditions described in Experimental. Dotted curves were extrapolations. K_m = Michaelis constant, S = substrate concentration (or substrate), V = velocity of reaction (mμmoles or smaller units/minute).

Experiment V

Studies on the 18-hydroxylation of 4-C^{14}-Corticosterone by fasciculata and glomerulosa mitochondrial fractions.

The generally low 18-hydroxylation activities reported in Experiments I (Tables V, VI), II (Fig. 10 (ii)), III (Table XV) and IV (Table XVIII), whether in the PIM (Experiment I), homogenate (Experiments I, II), slice (and quartered gland) (Experiments I, III), whole gland (Experiment III) or in sub-cellular mitochondrial fractions (Experiment IV) in presence of the 21-, and 11β-hydroxylating systems, and the relatively higher concentrations of 18-hydroxycorticosterone in the glomerulosa preparations had led to problems that need direct investigation of the 18-hydroxylation itself.

The glomerulosa and fasciculata mitochondrial preparations were used since 18-hydroxylation occured throughout the rat adrenal cortex (108 - 110) and the 18-hydroxylase system had been shown to be mainly associated with the mitochondrial fraction (4,000 g 10 minutes) (214 - 216, 49, 102) in the "heavy" mitochondria (216).

2. Experimental

60 adrenal glands from male rats were separated into glomerulosa and fasciculata (5) and directly homogenized in 18 ml. 0.44 M sucrose (Chapter IV (B) 1 (d), (e)). The mitochondrial fractions were obtained at 13,300 g.

The glomerulosa mitochondria were taken back finally in 3 ml. sucrose (0.44 M) while fasciculata mitochondria were dissolved finally in 15 ml. sucrose (0.44 M). 0.5 ml. of such glomerulosa was added to each of the five flasks A to E (equivalent to 10 glands per flask), and 0.25 ml. (equivalent to 1 gland) of the fasciculata mitochondria for each of the five flasks F to J. Flask K contained the mitochondrial fractions of 10 whole glands, while L contained the mitochondria of the fasciculata of 8 glands. All the incubation flasks containing 33.5 µmoles G-6-P, 4.8 µmoles NADP, 5 units G-6-P-D (disodium salt) and 1 mmole of malate each were adjusted to 3 ml. final volume with KRBG. The quantity of corticosterone was indicated in Table XIX and the specific activity of the 4-C^{14}-corticosterone precursor was 123,000 d.p.m. per mµmole. The vials were incubated for 30 minutes at 38°C..

After the addition of tritiated corticosterone and aldosterone as tracers, the incubates were extracted with dichloromethane. These extracts were processed for the percent conversion analyses as previously described (Chapter IV (B) 3, 4 and Figure 6).

2. Results and Discussions

The results obtained in this study are given in Table XIX and Figures 16 and 17 (pages 171, 175).

For better understanding of the results presented in this experiment, it should be noted that the protein content per gland in the glomerulosa tissue was 10 times smaller than that in the fasciculata tissue, as shown in Experiment IV. The glomerulosa mitochondrial fraction incubated in this experiment represented 10 glands, whereas the fasciculata mitochondrial fraction represented only 1 gland. The protein determination results indicated that those 10 glands contained 3047 µg. of protein for glomerulosa and that in the fasciculata was 2201 ug. of protein per flask in each case.

These values corresponded fairly well with those obtained in Experiment IV, as the following figures illustrated:

Experiment	µg. protein per gland	
	Glomerulosa	Fasciculata
IV	226	2176
V	305	2201

Hence the comparison of the 18-hydroxylating activities of the two zones on 4-C^{14}-corticosterone would be justifiable on similar total protein content basis.

In view of the very low conversion of the added precursor to 18-hydroxycorticosterone in Experiment IV, the incubation time in this experiment was prolonged, hoping to obtain higher conversion values for more valid comparisons. There was no preincubation of the tissues in this experiment prior to the homogenization, since a preincubation at 37°C.

was liable to cause some inactivation of the hydroxylases in these tissues. Sample K, containing mitochondrial fractions of 10 whole glands, served as a means of comparison.

In the photograph of the radioautograms of the chromatoplates on TLC - 1 (Fig.16) developed after 34 hrs. exposure, it can be seen that in the 5 samples A to E (glomerulosa mitochondrial fractions), there was a high percent conversions to aldosterone and 18-OH-B, but that in samples F to J, there were no conversion to aldosterone and only a slight conversion to 18-OH-B. Other unidentified compounds were formed in smaller percentages in the glomerulosa. Of these, one might be 11-dehydrocorticosterone.

When samples A and K were compared, sample A relative to the glomerulosa mitochondria and K to the whole gland of the rat adrenal. It was apparent that in K(from the whole gland), there was a smaller conversion to aldosterone and 18-OH-B than in sample A and more corticosterone was left unconverted in K (0.26 mμmoles) than in A (0.026 mμmoles)(Table XIX).

In sample L, where the mitochondrial fractions were from 8 glands, there was no conversion of corticosterone or only in traces that could not be detected. Thus, the

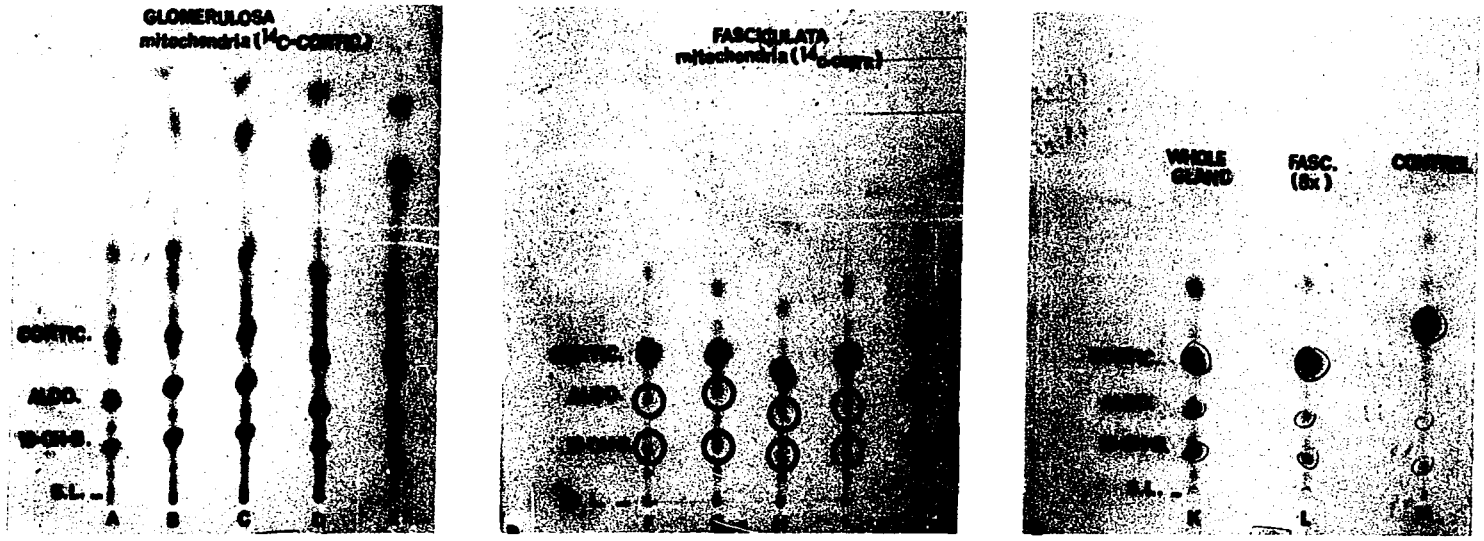


Fig. 16. Photocopy of the radioautograms taken of chromatograms on TLC - 1 (Table I) showing the incorporation of 4-C^{14}-corticosterone into different steroids when incubated glomerulosa, fasciculata (fasc.) and whole gland mitochondrial fractions of the rat adrenals. Incubation conditions were shown in Table XIX. S.L. = starting line (i.e., line of application on the chromatoplate).

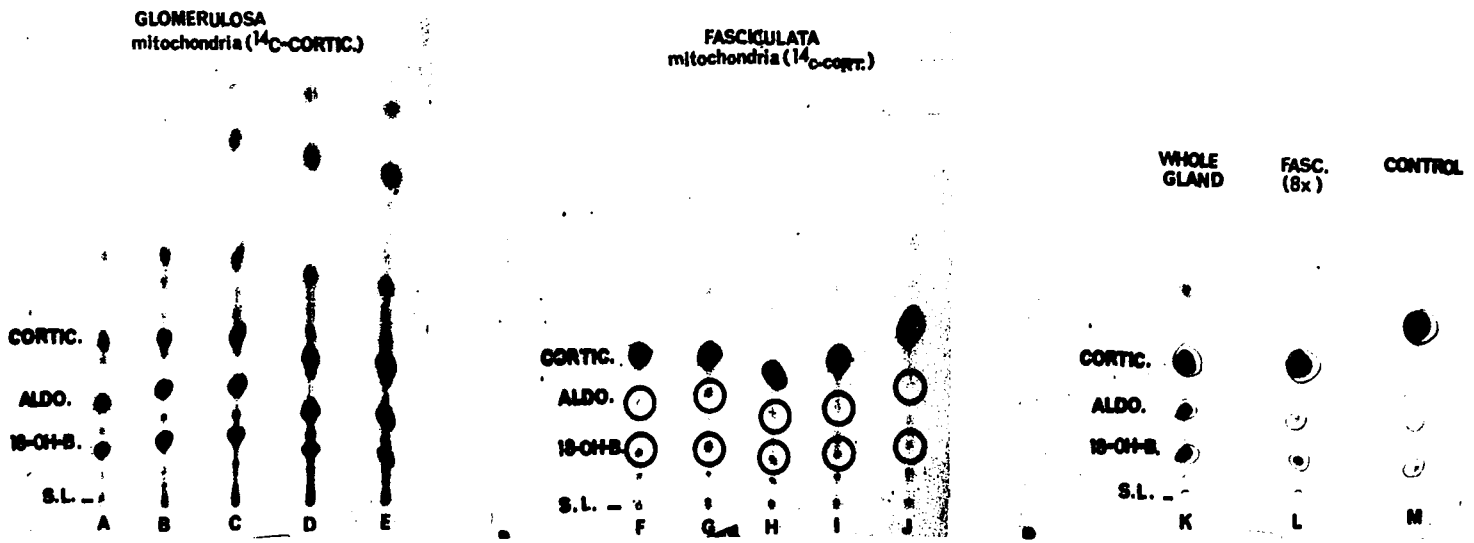


Fig. 16. Photocopy of the radioautograms taken of chromatograms on TLC - 1 (Table I) showing the incorporation of 4-C^{14}-corticosterone into different steroids when incubated glomerulosa, fasciculata (fasc.) and whole gland mitochondrial fractions of the rat adrenals. Incubation conditions were shown in Table XIX. S.L. = starting line (i.e., line of application on the chromatoplate).

conversion to 18-OH-B in fasciculata samples F to J was not due to the smaller amounts of tissues incubated, but, perhaps some inhibitory or unknown factors present.

Sample M is the control sample, where no mitochondrial fractionwas added. Only small amounts of impurities could be noted.

The quantitative results are given in Table XIX. The examination of this Table confirmed the points already underlined from the observations of the radioautograms (Fig. 16). Such quantitative data of Table XIX were indicatory that:

1. The high utilization of the labeled precursor by the glomerulosa mitochondria and the small utilization by the fasciculata mitochondria.

2. The lower conversion (almost 10 to 20 times) to 18-OH-B by the fasciculata mitochondria and the absence of aldosterone in samples F to J.

3. The relatively higher conversion to 18-OH-B than to aldosterone in the glomerulosa resulted in ratios of aldosterone to 18-OH-B (last column on the right of Table XIX) always around 0.7.

4. Under the present experimental conditions, the K_m for the 18-hydroxylation were not different to a great extent in the glomerulosa and fasciculata, being 1×10^{-7}M and 0.6×10^{-7}M respectively. Hence their differences in steroidogenic activities might be due to some other factors.

One of the important differences between the enzymatic systems of the glomerulosa and the fasciculata that could be detected at the mitochondrial level was that the 18-hydroxylation of corticosterone was inhibited in the fasciculata and not in the glomerulosa counterpart.

TABLE XIX

18-HYDROXYLATION OF 4-C^{14}-CORTICOSTERONE BY FASCICULATA AND GLOMERULOSA AND WHOLE GLAND MITOCHONDRIAL FRACTIONS

Tissue Preparations	Sample	Precursor added (mμmoles)	Precursor* Incorporated (mμmoles/30 mins.) 18-OH-B	Aldo.	mμmoles* Precursor Recovered	Ratio Aldo. / 18-OH-B
Glomerulosa	A	0.6	0.12	0.09	0.03	0.75
	B	1.2	0.27	0.18	0.06	0.65
	C	2.6	0.90	0.51	0.14	0.56
	D	6.1	1.62	1.23	0.35	0.77
	E	13.6	3.90	2.60	1.82	0.65
Fasciculata	F	0.6	0.006	-	0.52	-
	G	5.5	0.03	-	4.80	-
	H	13.9	0.09	-	12.60	-
	I	28.3	0.15	-	26.80	-
	J	142.6	0.52	-	131.20	-
10 whole glands	K	0.6	0.11	0.08	0.26	0.72
Fasciculata (8 times)	L	0.6	0	-	0.55	-
Control	M	0.6	0	0	0.60	-

Incubation conditions: 38° C. for 30 minutes under 95% O_2 - 5% CO_2 in KRBG

Specific activity of 4-C^{14}-Corticosterone: 123,000 d.p.m./mμmole

* calculated from percent radioactivity obtained after second TLC (TLC - 2).

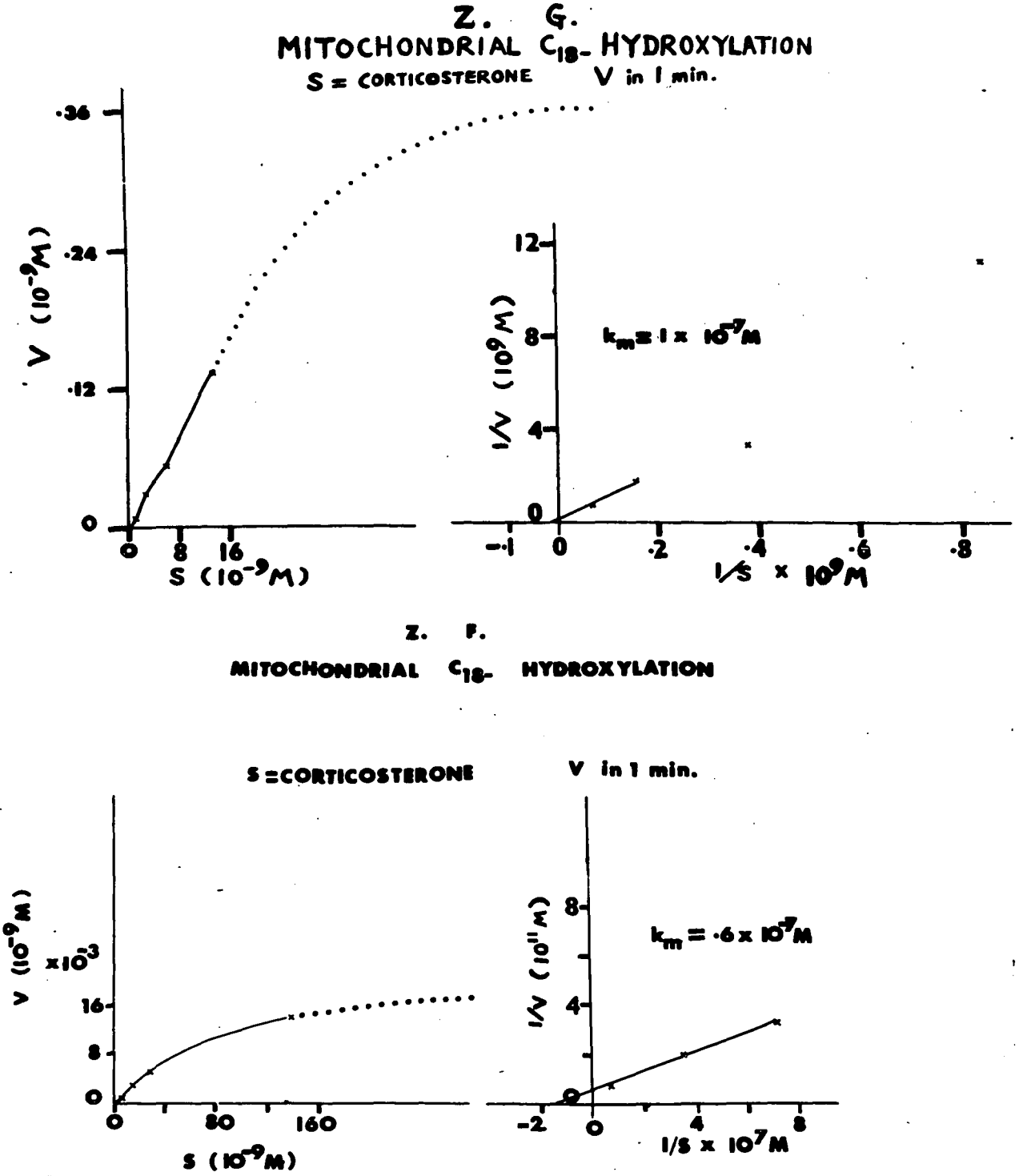


Fig. 17. Graphical presentation of the 18-hydroxylation data of Table XIX indicating the relative K_m values of the zona glomerulosa and fasciculata by the Lineweaver-Burk reciprocal plots.

CHAPTER VI

GENERAL DISCUSSIONS

A discussion and a conclusion of the results have already been given at the end of each experiment. In this Chapter, the significance and implications of the main findings in relation to the interpretation of the in vitro data will be discussed.

Radioactive precursors are used in in vitro work to clarify many points in the different steps involved in the pathway of steroid biogenesis. Although the in vitro systems are simplified compared to the in vivo investigations they are still very complex. In dealing with radioactive tracers, the homogenization of the added precursor with the endogenous precursor(s), the existence of one pool of enzymes in the tissue incubation, etc., must be assumed. More and more it appears that caution must be applied to the interpretation of the data thus obtained. Although the experiments conducted in vitro, unlike those in vivo, can be controlled, many factors inherent in the incubation itself can lead to artefacts which, when the investigator is not aware of them, can lead to erroneous interpretation of the data.

In this investigation it has been demonstrated that there are different pools of enzymes in the incubation of slices: namely the tissue and the medium itself. Indeed, the observation of TSANG that when the incubation media of rat adrenal slices are incubated in the absence of tissue (PIM) it is able to transform a steroid precursor such as progesterone into steroid products, mostly 11β-hydroxyprogesterone, if NADPH is available, has been confirmed and extended (39). The recognition of enzyme activity in the media itself raises the possibility that the incubation media may play an important role in the metabolism of added steroids in an incubation with slices. The results obtained in Expermient III seem in part to confirm this assumption. The very definite difference in the specific activity of the various steroids formed inside the tissue and in the media even after two hours of incubation could be interpreted as indicative of the existence of at least two pools - the media and the tissues - which do not readily mix.

However, since even the difference in specific activity can be seen in incubations of whole glands where the "leakage of the enzyme is minimal" (39), the tissue itself could be considered, as has been done by HALKERSTON (286), as constituted by different types of cells, the activity of which has been impaired in varying degrees. The membrane permeability

of the damaged cells could be changed in such a way that their penetration by the precursor is much more readily performed, as well as the release of their conversion products leading to a higher specific activity of the steroid released in the medium.

In an incubation of rat adrenal tissue these three possible pools of enzymes are not the only ones to be considered, since the adrenal gland is formed of different histological zones which have been demonstrated to produce different steroids, i.e., the glomerulosa produces aldosterone, 18-hydroxycorticosterone and corticosterone, very little 18-hydroxy,11-deoxycorticosterone; the fasciculata produces corticosterone and 18-hydroxy,11-deoxycorticosterone but not aldosterone, and very little 18-hydroxycorticosterone (152). Those results have been confirmed here. It has been demonstrated that the enzymatic activity of the mitochondrial fraction of glomerulosa tissue is very low compared to that of the fasciculata tissue on a weight or a gland basis. Moreover a substrate inhibition of the 11β-hydroxylase system with a relatively low concentration of 11-deoxycorticosterone has been found only in the mitochondrial fraction of the glomerulosa (Experiment IV),but in turn this same fraction can very easily hydroxylate in 18- position of the corticosterone molecule, whereas the fasciculata cannot (Experiment V). It

also seems that the 21-hydroxylation in the glomerulosa tissue is much smaller than in the fasciculata (Experiment I). These phenomena could explain in part the regulation of aldosterone formation: since the 21-hydroxylation is smaller, less 11-deoxycorticosterone is formed, leading to a small quantity of corticosterone, which is readily converted to aldosterone in the glomerulosa tissue but not in the fasciculata. However, the mechanism by which the 18-hydroxylation of 11-deoxycorticosterone occurs readily in the fasciculata but not the 18-hydroxylation of corticosterone, cannot be explained by this investigation. As has been indicated in Experiment III, the glomerulosa tissue can utilize the corticosterone and/or 18-hydroxy,11-deoxycorticosterone formed in the fasciculata tissue to produce more aldosterone and 18-hydroxycorticosterone when whole glands are incubated. In in vitro work generally the two zones are incubated together, as quartered glands. In this case there is a possible recirculation and utilization of the conversion product of the fasciculata zone by the glomerulosa zone. Since the 21-hydroxylase activity is very small in the glomerulosa, the production of 11-deoxycorticosterone and then of corticosterone is very low. The 18-hydroxylase system of this zone is not saturated and thus will be able to hydroxylate corticosterone and/or 18-hydroxy,11-deoxycorticosterone produced by the fasciculata. This recirculation of steroid produced can indeed lead to erroneous conclusions

when the action of trophic factors is looked for in vitro.

A puzzling phenomenon is the consistently very slight dilution of the radioactive progesterone by the endogenous progesterone (Experiments I, II and III). Is this the consequence of a poor penetration of the subcellular element by the added progesterone, or is it that progesterone may not be part of the normal sequence of corticosteroidogenesis? This is difficult to ascertain, but there are several reports as to the possibility that progesterone may be bypassed in this process (147, 158 - 160).

The concentration of the NADPH-generating system added to the media has to be considered, since it was shown that it is important in the overall metabolism of adrenal tissue (Experiments I and II). With the increasing concentration of this cofactor it was demonstrated that at relatively low concentrations the 21-hydroxylation first appears. If the concentration is increased the 11β- and 18-hydroxylations are activated. At a still higher concentration the NADPH is inhibitory not only to the 21-hydroxylase as reported by SHEPPARD (188) but also to the 11β- and 18-hydroxylating systems (Experiment II). There seems to be an inhibition of the general metabolism of the tissue (150) at the highest concentration. When added to slices the NADPH-generating system will not penetrate the membrane of normal cells

(287, 288). As HALKERSTON has demonstrated (286), it will act mostly on the damaged cells to increase, as has been shown in Experiment I, their endogenous production and, to a more limited extent, the conversion of the added precursor.

The duration of the incubation itself not only has a bearing on the quantitative aspect of the steroid production, but also on the qualitative aspect of this production. Indeed it seems that the 11β- and 18-hydroxylation of the steroid molecule occurs only when 11-deoxycorticosterone has been formed (Experiment II). This would mean that 11-deoxycorticosterone is a precursor in corticosteroidogenesis. 11β-hydroxyprogesterone seems to be formed only after a certain time as a consequence of a smaller 21-hydroxylase activity. This diminution in 21-hydroxylase activity has been demonstrated to be due in part to the inactivation of the 21-hydroxylase at 37°C. in dilute media (39)

The dilution of the homogenate with KRBG can have itself a marked effect on the endogenous production, as seen in Experiment I. This is at variance with the work of LAPLANTE and STACHENKO (289), where it was demonstrated that with an increase in the KRBG added to adrenal incubation slices, the endogenous production was not affected. The enzymes in slices are not diluted when more KRBG is added, whereas the concentration of enzyme/ml. is much smaller in

homogenates after dilution.

The quantity of precursor added can also have a bearing on the final results of the incubation. This precursor can have either an inhibitory (11-deoxycorticosterone in the glomerulosa, Experiment IV) or an activating (11-deoxycorticosterone in the fasciculata homogenate, Experiment II) effect. When the precursor concentration is small and is exhausted at the conclusion of the incubation, the percent conversion will not represent the potential capacity of the enzyme system to hydroxylate the added molecules. As has been found in Experiments I and II, the results will give an estimate of only the relative capacity of the different enzyme systems after a certain time.

In summary, the extrapolation of the conclusions obtained with in vitro data to in vivo processes is in general difficult, and before reaching a conclusion it is better to understand the action of factors which can influence the results in vitro, some of which have been outlined in this Thesis. However, the comparison of the results obtained with different tissue preparations, the use of quantitative methods (which, as the ones described here, measure the metabolism of the added precursor simultaneously with that of the endogenous precursor), the study of the kinetics of the different hydroxylations, will certainly

shed some light on the pathway of corticosteroidogenesis in the rat adrenal cortex.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Using double isotope methods developed for the simultaneous measurement of the specific activities and productions of steroids by different rat adrenal preparations incubated under varied conditions, it was demonstrated that:

(1) In the PIM, the hydroxylation of 4-C^{14}-progesterone occurred much more readily at the 11β- position, the 21- and 18-hydroxylations being very small.

(2) When no NADPH was added to the incubation of adrenal slices of either glomerulosa or fasciculata, the 21-hydroxylation of 4-C^{14}-progesterone was much higher than the 11β-hydroxylation.

(3) The addition of NADPH to the incubation media caused:
   (a) increases in all the hydroxylations of progesterone, but the 11β-hydroxylation was preponderant, especially in the glomerulosa.
   (b) the higher 11β-hydroxylation which was due to an accumulation of the 11β-hydroxyprogesterone.
   (c) a six to seven-time increase in the production of corticosterone, but the hydroxylation of 4-C^{14}-progesterone was enhanced by a factor of three only.

(4) The effect of malate was to inhibit the 21-hydroxylation of progesterone.

(5) The dilution of a fasciculata homogenate (30 mg./2 ml.) with 25 ml. KRBG increased the utilization of the added 4-C^{14}-progesterone by enhancing its 11β-, 18- and 21-hydroxylations. The endogenous production of corticosterone, however, was decreased by a factor of three.

(6) The dilution of a glomerulosa homogenate (15 mg./ml.) by a factor of 12 with KRBG increased the utilization of 4-C^{14}-progesterone, with a decrease in its 21- and 18-hydroxylations, and an increase in 11β-hydroxylation. The accumulation of the 11β-hydroxyprogesterone was high. Nevertheless, the endogenous production of corticosterone was unaffected.

(7) When only 2 ml. of the dilute homogenate was used with the same quantity of 4-C^{14}-progesterone, the utilization of 4-C^{14}-progesterone was comparatively higher. The 11β-hydroxylation was increased due to a higher accumulation of the 11β-hydroxyprogesterone by the glomerulosa homogenate. There was a decrease in the 21- and 18-hydroxylations, which were less marked in the fasciculata tissue.

(8) The reduction of the concentration of the substrate added did not affect the percentage of the hydroxylations.

(9) NADPH at a high concentration (23.2 μmoles/3 ml.) had an inhibitory action on the 21- and 18-hydroxylations.

(10) In either a dilute (1.5 mg./ml.) or concentrate (15 mg. per ml.) fasciculata homogenate, when the concentration of NADPH was below 23.2 mμmoles/3 ml., there was no hydroxylation of 4-C^{14}-progesterone. At a concentration of 23.2 mμmoles NADPH/3 ml., there was an activation of the 21-hydroxylation only; at the concentration of 232 mμmoles NADPH/3 ml., there were activations of the 21-, 18- and, more particularly, the 11β-hydroxylations. At still higher NADPH concentrations (2.32 μmoles or higher per 3 ml.), there were inhibitions in all the three hydroxylations.

(11) A time study in the corticosteroidogenesis by the dilute fasciculata homogenate demonstrated that DOC was produced first, followed by the 11β- and 18-hydroxylated products, corticosterone and 18-hydroxy,11-deoxycorticosterone. As the incubation continued, there were only slight increases in 21-hydroxylation and 11β-hydroxyprogesterone was accumulated.

<u>In an incubation with whole or quartered rat adrenal</u>:

(12) The specific activities of all the steroids studied were much smaller in the tissues than in the media.

(13) In an incubation, the specific activities of the conversion products were much lower than the final specific activity of 4-C^{14}-progesterone, the added precursor.

(14) Each of the steroids studied in this Thesis was present in significant concentration in the tissues.

(15) The concentrations of DOC and progesterone were much smaller in the media than in the tissues.

(16) The total amount of aldosterone and 18-hydroxy,11-deoxy-corticosterone produced by the whole glands (tissues and their respective media <u>together</u>) was almost twice the total amount produced by the quartered glands, under the same consideration.

(17) Although the quantity of corticosterone remained in the tissue was the same for either the quartered or whole glands, yet the quantity of corticosterone and 18-hydroxy, 11-deoxycorticosterone released into the medium by quartered glands was twice the amount released by the whole glands.

<u>In the study of the mitochondrial fractions of the glomerulosa and fasciculata</u>:

(18) The quantity of protein in the glomerulosa tissue was 10

times less than that in the fasciculata, and the 11β-hydroxylation activity in the glomerulosa was very low.

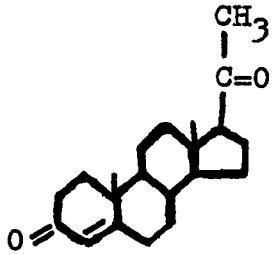
(19) A concentration of 43.8 mμmoles DOC/4 ml. was inhibitory for the 11β-hydroxylation by the glomerulosa mitochondria (4 glands).

(20) Only the glomerulosa mitochondria could hydroxylate the corticosterone molecule at the 18- position.

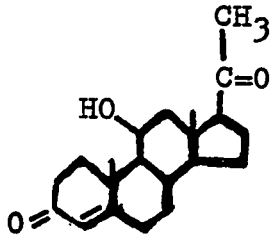
APPENDIX

APPENDIX A

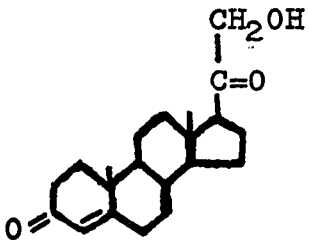
The structural formulae of the major steroids produced by the rat adrenal cortex studied in this Thesis:



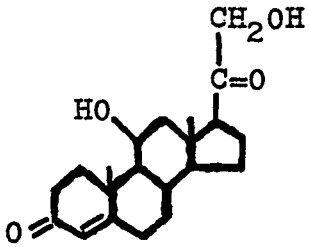
Progesterone



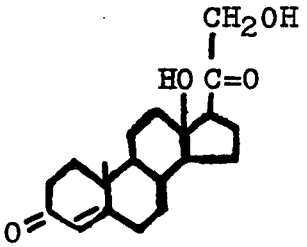
11β-Hydroxyprogesterone



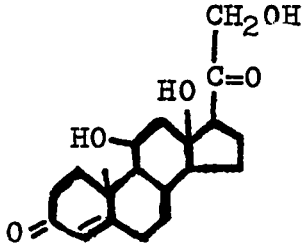
11-deoxycorticosterone



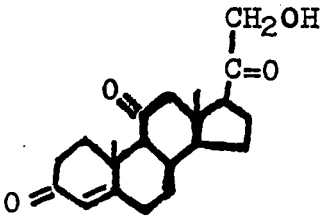
Corticosterone



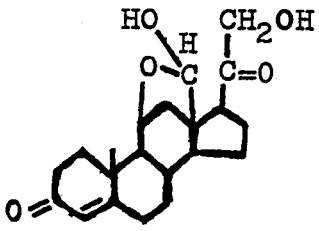
18-Hydroxy,11-deoxycorticosterone



18-Hydroxycorticosterone



11-Dehydrocorticosterone



Aldosterone
(Hemiacetal form)

BIBLIOGRAPHY

1. TSANG, C.P.W.; M.Sc. Thesis, McGill University, Montreal, 1965.

2. TSANG, C.P.W. and A. CARBALLEIRA; Proc. Soc. Exptl. Biol. and Med. 122: 1031, 1966.

3. SCHÖNBAUM, E., M. DAVIDSON, R.E. LARGE and W.G. CASSELMAN; Can. J. Biochem. Physiol. 37: 1209, 1959.

4. TSANG, C.P.W. and J. STACHENKO; Can. J. Biochem. 47: 1109, 1969.

4a. HALKERSTON, I.D.K., M. FEINSTEIN and O. HECHTER; Fed. Proc. 25: 494, 1966 (Abstract).

5. GIROUD, C.J.P., J. STACHENKO and E. H. VENNING; Proc. Soc. Exptl. Biol. and Med. 92: 154, 1954.

6. SANCTOSEVERINATUS, B.E.; "De Glandulis Quae Renibus Incumbunt", 1563.

7. ADDISON, T.; The Constitutional and Local Effects of Disease of the Suprarenal Capsules. London: S. Highley, 1855.

8. BROWN-SEQUARD, C.E.; Arch. Gen. de Med. 8: 385, 1856.

9. THORN, G.W. and P.H. FORSHAM; The Adrenals Williams Textbook of Endocrinology. London: Saunders, 1950.

10. HAM, A.W. and T.S. LEESON; Histology (4th ed.). Phila., Montreal: Lippincott, 1961. 763 pp.

11. ARNOLD, J.; Virschow's Arch. 35: 64, 1866.

12. FAWCETT, D.W., J.A. LONG and A.L. JONES; Rec. Prog. Horm. Res. 25: 315, 1969.

13. ROGOFF, J.M. and G.N. STEWART; J.A.M.A. 92: 1569, 1929.

14. HENCH, P.S., E.C. KENDALL, C.H. SLOCOMBE and H.F. POLLEY; Proc. Staff Meetings, Mayo Clinic 24: 181, 1949.

15. HECHTER, O. and G. PINCUS; Physiol. Revs. 34: 459, 1954.

16. GRANT, J.K. In: "Biosynthesis and Secretion of Adrenocortical Steroids" Biochem. Society Symposia No. 18. Cambridge, 1960. 24 pp.

17. GRANT, J.K.; Brit. Med. Bull. 18: No. 2, 1962.

18. YATES, F.E. and J. URQUHART; Physiol. Rev. 42: 359, 1962.

19. VOGT, M.; J. Physiol. 102: 341, 1943.

20. MCDONALD, I.R., J.R. GODING and R.D. WRIGHT; Aust. J. Expt. Biol. Med. Sci. 36: 83, 1958.

21. HARRISON, R.G.; Anat. Rec. 1: 116, 1907.

22. CARREL, A.: J. Exptl. Med. 38: 407, 1923.

23. HECHTER, O., A. ZAFFARONI, R.P. JACOBSEN, H, LEVY, R.W. JEANLOZ, V. SCHENKER and G. PINCUS; "The Nature and the Biogenesis of Adrenal Secretory Product" Rec. Prog. Horm. Res. 6: 215, 1951.

24. HECHTER, O., R.P. JACOBSEN, V. SCHENKER, H, LEVY, R.W. JEANLOZ, C.W. MARSHALL, G. PINCUS; Endoc. 52: 679, 1953.

25. WARBURG, O.; Über den Stoffwechsel der Tumoren Springer, Berlin. (English translation by F. Dickens, London: Constable, 1930.)

26. SAFFRAN, M., B. GRAD and M.J. BAYLISS; Endoc. 50: 639, 1952.

27. SAFFRAN, M. and M.J. BAYLISS; Endoc. 52, 142, 1953.

28. _____, ibid., 52: 140, 1953.

29. STACHENKO, J. and C.J.P. GIROUD; Can. J. Biochem. 42: 1777, 1964.

30. HAYANO, M and R.I. DORFMAN; J. Biol. Chem. 201: 175, 1953.

31. PERON, F.G. and J.L. MCCARTHY; "Corticosteroidogenesis in the Rat Adrenal Gland" in Functions of Adrenal Cortex. K.W. McKerns, ed. New York (Division of Meredith Corp.): Appleton-Century-Crofts, 1968. 263 pp.

32. RYAN, K.J. and L.L. ENGEL; J. Biol. Chem. 225: 103, 1957.

33. SAFFRAN, M., P. FORD, E.K. MATTHEWS, M. KRAML and L. GARBACZEWSKA; Can. J. Biochem. 45: 1901, 1967.

34. TAIT, S.A.S., J.F. TAIT, M. OKAMOTO and C. FLOOD; Endocrinology 81: 1213, 1967.

35. ELLIOTT, K.A.C.; Methods in Enzymology. Vol I New York: Academic Press, 1955. 9 pp.

36. POTTER, V.R. and C.A. ELVENHJEM; J. Biol. Chem. 114: 495, 1936.

37. POTTER, V.R.; Methods in Enzymology. Vol. I New York: Academic Press, 1955.

38. UMBREIT, N.W., R.H. BURRIS and J.F. STAUFFER; "Manometric Techniques and Tissue Metabolism" (2nd printing). Minneapolis 15 (Min.): Burgess Publishing Co., 1959.

39. TSANG, C.P.W.; Ph. D. Thesis, McGill University, Montreal, 1968.

40. SCHNEIDER, W.C. and G.H. HOGEBOOM; Cancer Res. 11: 1, 1951.

41. HOGEBOOM, G.H., W.C. SCHNEIDER and G.E. PALADE; J. Biol. Chem. 172: 619, 1948.

42. CAMMER, W. and R.W. ESTABROOK; Arch. Biochem. Biophys. 122: 636, 1967.

43. PERON, F.G. and T.L. MCCARTHY; "Corticosteroidogenesis in the Rat Adrenal Gland" in Functions of Adrenal Cortex. K.W. McKerns, ed. New York (Division of Meridith Corp.): Appleton-Century-Crofts, 1968. 270 pp.

44. GREEN, D.E.; Mechanisms of Biological Oxidations. London: Cambridge University Press, 1940.

45. SUMNER, J.B. and G.F. SOMNERS; Chemistry and Method of Enzymology (2nd ed.). New York: Academic Press, 1947.

46. COLOWICK, S.P. and N.O. KAPLAN, eds.; Methods in Enzymology. Vols. 1, 2. New York: Academic Press, 1955.

47. COLOWICK, S.P. and N.O. KAPLAN, eds.; Methods in Enzymology. Vol. 3. New York: Academic Press, 1956.

48. GRANT, J.K.; Biochem. J. 64: 559, 1956.

49. RAMAN, P.B., D.C. SHARMA and R.I. DORFMAN; Biochem. 5: 1795, 1966.

50. SHARMA, D.C., E. FORCHIELLI and R.I. DORFMAN; J. Biol. Chem. 237: 1495, 1962.

51. UMBREIT, N.W., R.H. BURRIS and J.F. STAUFFER; "Manometric Techniques and Tissue Metabolism" (2nd printing) Minneapolis 15 (Min.): Burgess Publishing Co., 1959. 219 pp.

52. KLYNE, W.; The Chemistry of Steroids. Peters, R. and F.G. Young, eds. Methuen's Monographs on Biochemical Subjects, 1957.

53. WEINSTEIN, L.H. and H.J. LAURENCOT Jr.; Instrumental Methods of Experimental Biology. D.W. Newman, ed. New York: Macmillan Co., London: Collier-Macmillan Ltd., 1967. 113 pp.

54. BURTON, R.B., A ZAFFARONI and E.H. KEUTMAN; J. Biol. Chem. 188: 763, 1951.

55. ZAFFARONI, A. and R.B. BURTON; J. Biol. Chem. 193: 749, 1951.

56. SAVARD, K.; J. Biol. Chem. 202: 457, 1953.

57. BUSH, I.E.; Brit. Med. Bull. 10: 231, 1954.

58. EBERLEIN, W.R. and A.M. BONGIOVANNI; Arch. Biochem. Biophys. 59: 90. 1955.

59. LISBOA, B.P.; Acta Endoc. 43: 47, 1963.

60. STAHL, E., ed. Thin-layer Chromatography. New York: Academic Press, 1965.

61. COX, J.S.G., L.B. HIGH and E.R.H. JONES; Proc. Chem. Soc. 234, 1958.

62. KLYNE, W.; The Chemistry of Steroids. Peters, R. and F.G. Young, eds. Methuen's Monographs on Biochemical Subjects, 1957. 186 pp.

63. FIESER, L.F. and M. FIESER; Steroids. New York: Reinhold Publishing Corp., London: Chapman and Hall Ltd., 1959. 16 p.

64. ELLIOTT, F.G., M.K.BIRMINGHAM, A.V. SCHALLY and E. SCHONBAUM; Endocrinology 55: 722, 1954.

65. UMBERGER, E.J.; Analyt. Chem. 27: 768, 1955.

66. SINGER, B. and M.P. STACK-DUNNE; J. Endoc. 12: 130, 1955.

67. PERON, F.G.; Endocrinology 66: 458, 1960.

68. MADOR, W.J. and R.R. BUCK; Anal. Chem. 24: 666, 1952.

69. FIESER, L.F. and M. FIESER; Steroids. New York: Reinhold Publishing Corp., London: Chapman and Hall, Ltd., 1959. 632 pp.

70. NORYMBERSKI, J.K., R.D.STUBBES and H.F. WEST; Lancet 1: 1276, 1953.

71. _____, ibid.; J. Biol. Chem. 60: 453, 460, 1955.

71a. _____, ibid.; J. Biol. Chem. 64: 168, 1956.

72. BROOKS, C.J.W. and J.K. NORYMBERSKI; Biochem. J. 55: 371, 1953.

73. KLYNE, W.; The Chemistry of the Steroids. Peters, R. and F.G. Young, eds. Methuen's Monographs on Biochemical Subjects, 1957. 140 pp.

74. ZIMMERMANN, W.; Z. Physiol. Chem. 233: 257, 1935.

75. PORTER, C.C. and R.H. SILBER; J. Biol. Chem. 185: 201, 1950.

76. ZAFFARONI, A. and R.B. BURTON; J. Biol. Chem. 193: 749, 1951.

77. KLYNE, W.; The Chemistry of the Steroids. Peters, R. and F.G. Young, eds. Methuen's Monographs on Biochemical Subjects, 1957. 71 p.

78. KLIMAN, B. and R.E. PETERSON; J. Biol. Chem. 235, 1639, 1960.

79. ZAFFARONI, A.; Rec. Prog. Horm. Res. 8: 51, 1953.

80. MAYO, P. de and R.I. REED; Chem. Ind. 1481, 1956.

81. CALLOW, R.K. and F.G. YOUNG; Proc. Roy. Soc., London. A 157: 194, 1936.

82. DOBRINER, K., E.R. KATZENELLENBOGEN and R.N. JONES; Infrared Absorption Spectra of Steroids. Vol. I New York: Interscience Publishers, 1953.

83. ROBERTS, G., B.S. GALLAGHER and R.N. JONES; Infrared Absorption Spectra of Steroids. Vol. II New York: Interscience Publishers, 1958.

84. JONES, R.N.; J. Org. Chem. 19: 1252, 1954.

85. FIESER, L.F. and M. FIESER; Steroids. New York: Reinhold Publishing Corp., London: Chapman and Hall Ltd., 1959. 185 p.

86. FLETT, M. St.; Physical Aids to the Organic Chemist. New York -Amsterdam: Elsevier Publish. Co., 1962. 255 pp.

87. PARSONS, J., W. BEHER and G.D. BAKER; Anal. Chem. 27: 514, 1569, 1955.

88. _____, ibid., 28: 1514, 1956.

89. _____, ibid., 29: 762, 1957.

90. How to use the Radioisotopic Derivative Methods in Quantitative Analyses. (Technical Bulletin No. 3, 1958) Nuclear Chicago Corp., U.S.A..

91. GORSUCH, T.T.; "Radioactive Tracers in Chemical Analysis" in U.K.A.E.A. R.C.C. Review No. 5 (October, 1966). Amersham, Bucks, England: The Radiochemical Centre.

92. Selected References to Tracer Techniques (revised ed.) in U.K.A.E.A. R.C.C. Review I (2nd. ed. 1965). Amersham, Bucks, England: The Radiochemical Centre.

93. "Radioactive Isotope Dilution Analysis" in U.K.A.E.A. R.C.C. Review No. 2 (2nd. ed. 1965). Amersham, Bucks, England: The Radiochemical Centre.

94. PETERSON, R.E.; Symposium on Advances in Tracer Applications of Tritium, October 31, 1958, New York City. Sponsors: New England Nuclear Corp., Packard Instrument Co., Inc., and Atomic Associates.

95. STACHENKO, J., C. LAPLANTE and C.J.P. GIROUD; Can. J. Biochem. 42: 1275, 1964.

96. PFIFFNER, J.J., O. WINTERSTEINER and H.M. VARS; J. Biol. Chem. 111: 585, 1935.

97. KENDALL, E.C., M.L. MASON, W.M. HOEHN and B.F. MCKENZIE; Proc. Staff Meetings Mayo Clinic 12: 136, 270, 1937.

98. MASON, M.L., W.M. HOEHN and E.C. KENDALL; J. Biol. Chem. 124: 459, 1938.

99. REICHSTEIN, T. and C.W. SHOPPEE; Vitamins and Hormones 1: 346, 1943.

100. HAYANO, M., N. SABA, R.I. DORFMAN and O. HECHTER; Rec. Prog. Horm. Res. 12: 79, 1956.

101. BUSH, I.E.; Ciba Foundation Colloquia on Endocrinology. Vol. 7 London: Churchill, 1953. 210 p.

102. SANDOR, T.; General and Comparative Endocrinology. Suppl. 2, January, 1969. 284 pp.

103. BUSH, I.E.; J. Physiol. 115: 12P, 1951.

104. VOGT, J.; J. Physiol. 130: 601, 1955.

105. WARD, P.J. and M.K. BIRMINGHAM; Biochem. J. 76: 269, 1960.

106. PERON, F.G.; J. Biol. Chem. 236: 1764, 1961.

107. SIMPSON, S.A., TAIT J.F. and I.E. BUSH; Lancet 2: 226,1952.

108. BIRMINGHAM, M.K. and P.J. WARD; J. Biol. Chem. 236: 1661, 1961.

109. PERON, F.G.: Endocrinology 69: 39, 1961.

110. PERON, F.G.; Program of the 43rd. Meeting of Endocrine Society, Abstract No. 82, 1961.

111. SANDOR, T. and A. LANTHIER; Acta Endoc. 42: 355, 1963.

112. LUCIS, R., A. CARBALLEIRA and E.H. VENNING; Steroids 6: 737, 1965.

113. HECHTER, O. and G. G. PINCUS; Physiol. Revs. 34: 460, 1954.

114. HEARD, R.D.H., E.G. BLIGH, M.C. CANN, P.H. JELLINCK, V.J. O'DONNELL and B.G. RAO and J.L. WEBB; Rec. Prog. Horm. Res. 12: 45, 1956.

115. SRERE, P.A., I.L. CHAIKOFF and W.G. DAUBEN; J. Biol. Chem. 176: 8291, 1948.

116. ZAFFARONI, A., O. HECHTER and G. PINCUS; J. Am. Chem. Soc. 73: 1390, 1951.

117. HAYNES, R., K. SAVARD and R.I. DORFMAN; Science 116: 690, 1952.

118. HECHTER, O., M.M. SOLOMON, A. ZAFFARONI and G. PINCUS; Arch. Bioch. and Biophys. 46: 201, 1953.

119. BLOCH, K. and D. RITTENBERG; J. Biol. Chem. 155: 243, 1944.

120. HECHTER, O.; "Cholesterol" R.P. Cook, ed. New York: Academic Press, 1958. 309 pp.

121. STONE, D. and O. HECHTER; Arch. Biochem. Biophys. 51: 457, 1954.

122. WERBIN, H. and I.L. CHAIKOFF; Arch. Bioch. Biophys. 93: 476, 1961.

123. SAYERS, G., M.A. SAYERS, E.G. FRY, A. WHITE and C.N.H. LONG; Yale J. Biol. Med. 16: 361, 1944.

124. BLOCH, E. and K. BENIRSCHKE; J. Biol. Chem. 234: 1085, 1959.

125. WERBIN, H., I.L. CHAIKOFF and E.E. JONES; J. Biol. Chem. 234: 282, 1959.

126. GOODMAN, D.S., J. AVIGAN and H. WILSON; J. Clin. Invest. 41: 2135, 1962.

127. BLOCH, K.; Science 150: 19, 1965.

128. HECHTER, O., R.P. JACOBSEN, R.W. JEANLOZ, H. LEVY, C.W. MARSHALL, G. PINCUS and V. SCHENKER; J. Am. Chem. Soc. 71: 3261, 1949.

129. ZAFFARONI, A., O. HECHTER and G. PINCUS; Fed. Proc. 10: 150, 1951.

130. CASPI, E., R.I. DORFMAN, B.T. KHAN, G. ROSENFELD and W. SCHMID; J. Biol. Chem. 237: 2085, 1962.

131. LYNN, W.S.,Jr., E. STAPLE and S. GURIN; Fed. Proc. 14: 783, 1955.

132. LYNN, W.S., Jr., E. STAPLE and S. GURIN; J. Am. Chem. Soc. 76: 4048, 1954.

133. CHENG, S.C.; Ph. D. Thesis, McGill University, Montreal, 1969.

134. OMURA, T., R. SATO, D.Y. COOPER, O. ROSENTHAL and R.W. ESTABROOK; Fed. Proc. 24: 1181, 1965.

135. LIEBERMAN, S., L. BRANDY, V. LIPPMAN and K.D. ROBERTS; Bioch. Biophys. Res. Commun. 34: 367, 1969.

136. ROBERTS, K.D., L. BANDI, H.I. CALVIN, W.D. DRUCKER and S. LIEBERMAN; J. Am. Chem. Soc. 86: 958, 1964.

137. CALVIN, H.I. and S. LIEBERMAN; Biochem.3: 259, 1964.

138. CONSTANTOPOULOS, G., P.S. SATOCH and T.T. TCHEN; Bioch. Biophys. Res. Commun. 8: 50, 1962.

139. SAMUELS, L.T.; Ciba Colloquia on Endocrinology. Vol. 7. London: Churchill, 1953. 176 p.

140. SAMUELS, L.T., M.L. HELMREICH, M.B. LASATER and Y. REICH; Science 113: 490, 1951.

141. TALALAY, P.; Physiol. Rev. 37: 362, 1957.

142. EWALD, W., H. WERBIN and I.L.CHAIKOFF; Steroids 3: 505, 1964.

143. KRUSKEMPER, H.L., E. FORCHIELLI and H.L. RINGOLD; Steroids 3: 295, 1964.

144. TALALAY, P. and V.S. WANG; Bioch. Biophys. Acta 18: 300, 1955.

145. KAWARHARA, F.S., V.S. WANG and P. TALALAY; J. Biol. Chem. 237: 1500, 1962.

146. KOWAL, J.E., E. FORCHIELLI and R.I. DORFMAN; Steroids 4: 77, 1964.

147. KOWAL, J. E., E. FORCHIELLI and R.I. DORFMAN; Steroids 3: 531, 1964.

148. EICHHORN, J. and O. HECHTER; Proc. Soc. Exptl. Biol. and Med. 97: 614, 1958.

149. HECHTER, O. and G. PINCUS; Physiol. Revs. 34: 482, 1954.

150. BROWNIE, A.C., J.K. GRANT and D.W. DAVIDSON; Biochem. J. 58: 218, 1954.

151. GIROUD, C.J.P., J. STACHENKO and P. PILETTA; International Symposium on Aldosterone. Boston: Little Brown and Co., publishers, 1958. 56 p.

152. STACHENKO, J. and C.J.P. GIROUD; Can. J. Biochem. 42: 1784, 1964.

153. KRAULIS, I. and M.K. BIRMINGHAM; Acta Endoc. 47: 79, 1964.

154. MAKOFF, R., S. ROBERTS and D.D. FOWLER; J. Biol. Chem. 239: 4124, 1964.

155. CREANGE, J.E. and S. ROBERTS; Steroids - Suppl. II 13, 1965.

156. DORFMAN, R.I., M. HAYANO, R. HAYNES and K. SAVARD; Ciba Foundation Colloquia on Endocrinology. Vol. 7 London: Churchill, 1953. 191 p.

157. SHIMIZU, K.; Steroid Dynamics. Pincus, G., T. Nakao and J.F. Tait, eds. New York and London: Academic Press, 1966. 481 p.

158. WELIKY, I. and L.L. ENGEL; J. Biol. Chem. 237: 2089, 1962.

159. BERLINGER, D.L., D.M. CAZES and C.J. NABORS; J. Biol. Chem. 237: 2478, 1962.

160. PASQUALINI, J.R., G. LAFOSCADE and M.F. JAYLE; Steroids 4: 739, 1964.

161. WHITEHOUSE, B.J. and G.P. VINSON; Steroids 245, 1968.

162. AYRES, P.J., J. EICHHORN, O. HECHTER, N. SABA, J.F. TAIT and S.A.S. TAIT; Acta Endoc. 33: 27, 1960.

163. SHEPPARD, H., R. SWENSON and T.F. MOWIES; Endocrinology 73: 819, 1963.

164. CHEN, P.S., Jr., H.P. SCHEDI, G. ROSENFELD and F.C. BARTTER; Proc. Soc. Exptl. Biol. (N.Y.) 97: 683, 1958.

165. WETTSTEIN, A., F.W. KAHNT and R. NEHER; Ciba Foundation Colloquia on Endocrinology 8: 160, 1954.

166. TRAVIS, R.H. and G.H. FARRELL; Endocrinology 63: 832, 1958.

167. AYRES, P.J., R. GOULD, S.A. SIMPSON and J.F. TAIT; Biochem. J. 63: 19P, 1956.

168. AYRES, P.J., O. GARROD, S.A.S. TAIT and J.F. TAIT In: An International Symposium on Aldosterone. Muller A.F. and C.M. O'Connor, eds. London: Churchill, 1958. 143 p.

169. HARDING, B.W. and D. NELSON; J. Biol. Chem. 241: 2212, 1966.

170. KAHNT, F.W., and R. NEHER; Helv. Chim. Acta 48: 1457, 1965.

171. RAMAN, P.B., R.J. ERTEL and F. UNGAR; Endocrinology 74: 865, 1964.

172. KRAULIS, I. and M.K. BIRMINGHAM; Acta Endoc. (Kobenhavn) 47: 514, 1964.

173. NICOLA, A.F. de, H. TRAIKOV and M.K. BIRMINGHAM; Endocrinology 84: 104, 1969.

174. NEHER, R. and A. WETTSTEIN; Helv. Chim. Acta 43: 623, 1960.

175. ULICK, S. and K. KUSCH; J. Am. Chem. Soc. 82: 6421, 1960.

176. NICOLIS, G. and S. ULICK; Endocrinology 76: 514, 1965.

177. STACHENKO, J. and C.J.P. GIROUD; International Symposium o Adrenal Cortex and Adrenal Cortical Hormones. Ghent, 1962.

178. ULICK, S., E. GAUTIER, K.K. VETTER, J.R. MARKELLO, S. YAFFE and C.U. LOWE; J. Clin. Endoc. 24: 669, 1964.

179. PASQUALINI, J.R.; Nature 201: 501, 1964.

180. PSYCHOYOS, S., H.H. TALLAN and P. GREENGARD; J. Biol. Chem. 241: 2949, 1966.

181. TALLAN, H.H., S. PSYCHOYOS and P. GREENGARD; J. Biol. Chem. 242: 1912, 1967.

182. GREENGARD, F.W., S. PSYCHOYOS, H.H. TALLAN, D.Y. COOPER, O. ROSENFELD and R.W. ESTABROOK; Arch. Bioch. Biophys. 121: 298, 1967.

183. RYAN, K.J. and L.L.ENGEL; J. Biol. Chem. 225: 103, 1957.

184. ROSENTHAL, O. and S. NARASIMHULU; Methods in Enzymology. Vol 15 R.B. Clayton, ed. New York and London: Academic Press, 1969. 596 p.

185. STACHENKO, J. and C.J.P. GIROUD; Endoc. 64: 730, 1959.

186. _____, ibid., 743 pp.

187. ROSENTHAL, O. and S. NARASIMHULU; Methods in Enzymology. Vol. 15 R.B. Clayton, ed. New York and London: Academic Press, 1969. 628 p.

188. SHEPPARD, H., J.N. BEASELEY and J.L. WACKER; Fed. Proc. 25: 551, No. 1040, 1966.

189. TSANG, C.P.W. and J. STACHENKO; Proc. Can. Fed. Bio. Soc. 10: 64, 1967.

190. BRYSON, M.J. and M.L. SWEAT; J. Biol. Chem. 243, 2799, 1968.

191. HAYANO, M., R.I. DORFMAN and E.Y. YAMADA; J. Biol. Chem. 193: 175, 1951.

192. HAYANO, M. and R.I. DORFMAN; J.Biol. Chem. 211: 227, 1954.

193. SWEAT, M.L.; J. Am. Chem. Soc. 73: 4056, 1951.

194. SWEAT, M.L. and M.D. LIPSCOMB; J. Am. Chem. Soc. 77: 5185, 1955.

195. BROWNIE, A.C. and J.K. GRANT; Bioch. J. 57: 255, 1954.

196. BROWNIE, A.C. and J.K. GRANT; Bioch. J. 62: 29, 1956.

197. HARDING, B.W., L.D. WILSON , S.H. WONG and D.H. NELSON; Steroids, Suppl. II 51, 1965.

198. TOMKINS, G.M., P.J. MICHAEL and J.F. CURRAN; Bioch. Biophys. Acta 23: 655, 1957.

199. GORBMAN, A. and H.A. BERN; A Textbook of Comparative Endocrinology. New York: John Wiley & Sons, Inc., 1962.

200. GASKELL, J.F.; J. Gen. Physiol. 2: 73, 1919.

201. ROAF, H.E. and M.J. NIERENSTEIN; J. Physiol. (London) 36: V, 1907.

202. OSTLUND, E.; Acta Physiol. Scand. 31: (Suppl. 112), 1954.

203. SWEAT, M.L. and M.J. BRYSON; Arch. Biochem. Biophys. 96: 186, 1962.

204. TOMKINS, G.M., J.F. CURRAN and P.J. MICHAEL; Biochem. Biophys. Acta 28: 449, 1958.

205. SUZUKI, K. and T. KIMURA; Biochem. Biophys. Res. Commun. 19: 340, 1965.

206. SWEAT, M.L. and R.B. YOUNG; Fed. Proc. 25: 185, 1966.

207. HAYANO, M.,N. SABA, R.I. DORFMAN and O. HECHTER; Rec. Prog. Horm. Res. 12: 113, 1956.

208. _____, ibid., 59 p.

209. COOPER, D.Y. and O.ROSENTHAL; Arch. Biochem. Biophys. 96: 331, 1962.

210. _____, ibid., 96 p.

211. SWEAT, M.L., and M.J. BRYSON; Endocrinology 76: 773, 1965.

212. PERON, F.G. and J.L. MCCARTHY; "Corticosteroidogenesis in the Rat Adrenal Gland" in Functions of Adrenal Cortex. K.W. McKerns, ed. New York (Division of Meredith Corp.): Appleton-Century-Crofts, 1968. 273 p.

213. SHARMA, D.C., E. FORCHIELLI and R.I. DORFMAN; J. Biol. Chem. 238: 572, 1963.

214. PSYCHOYOS, S., H.H. TALLAN and P. GREENGARD; Fed. Proc. 24: 449, 1965.

215. NAKAMURA, Y. and B.TAMAOKI; Biochem. Biophys. Acta 85: 350, 1964.

216. WILSON, L D., D.H. NELSON and R.W. HARDING; Biochem. Biophys. Acta 99: 391, 1965.

217. HAYANO, M. and R.I. DORFMAN; Arch. Biochem. Biophys. 59: 531, 1957.

218. SWEAT, M.L., R.A. ALDRICH, C.H. De BRUIN, W.C. FOWLKS, L.R. HEISELT and H.S. MASON; Fed. Proc. 15: 367, 1956.

219. HAYANO, M., A. SAITO, D. STONE and R.I. DORFMAN; Biochim. et Biophys. Acta 21: 381, 1956.

220. HAYANO, M., R.I. DORFMAN and E. ROSEMBERG; Fed. Proc. 14: 224, 1955.

221. SWEAT, M.L. and M.D. LIPSCOMB, quoted in (220).

222. HALKERSTON, I.D.K., J. EICHHORN and O. HECHTER; Arch. Biochim. Biophys. 85: 287, 1959.

223. HALKERSTON, I.D.K., J. EICHHORN and O. HECHTER; Fed. Proc. 19: 160, 1960.

224. PERON, F.G. and J.L. MCCARTHY; "Corticosteroidogenesis in Rat Adrenal Gland" in Functions of Adrenal Cortex. K.W. McKerns, ed. New York (Division of Meridith Corp.): Appleton-Century-Crofts, 1968, 287 p.

225. HALKERSTON, I.D.K., J. EICHHORN and O. HECHTER; J. Biol. Chem. 236: 380, 1961.

226. PERON, F.G.and J.L. MCCARTHY; "Corticosteroidogeneeis in the Rat Adrenal Gland" in Functions of Adrenal Cortex. K.W. McKerns, ed. New York (Division of Meredith Corp.): Appleton-Century-Crofts, 1968. 302 p.

227. HARDING, B.W. and D.H. NELSON; Endocrinology 75: 501, 1964.

228. _____, ibid., 506 pp.

229. PURVIS, J.L.; Biochem. Biophys. Acta 38: 435, 1960.

230. PERON, F.G. and J.L. MCCARTHY; "Corticosteroidogenesis in the Rat Adrenal Gland" in Functions of Adrenal Cortex. K.W. McKerns, ed. New York (Division of Meredith Corp.): Appleton-Century-Crofts, 1968. 303 p.

231. HECHTER, O. and G. PINCUS; Physiol. Revs. 34: 351, 1954.

232. PERON, F.G., J.L. MCCARTHY and F. GUERRA; Biochim. et Biophys. Acta 117: 450, 1966.

233. GUERRA, F., F.G. PERON and J.L. MCCARTHY; Biochim. et Biophys. Acta 117: 433, 1966.

234. PERON, F.G.and J.L. MCCARTHY; Biochim. et Biophys. Acta 117: 454, 1966.

235. SIH, J.J., Y.Y. TSONG and B. STEIN; J. Am. Chem. Soc. 90: 5301, 1968.

236. HAYNES, R.C.,Jr. and L. BERTHET; J. Biol.Chem. 225: 115, 1957.

237. GLOCK, G.E. and P.MCLEAN; Bioch. J. 56: 171, 1954.

238. KELLY, T.L., E.D. NIELSON, R.B. JOHNSON and C.C. VESTING; J. Biol. Chem. 212: 545, 1955.

239. COHEN, R.B.; Proc. Soc. Exptl. Biol. Med. 101: 405, 1959.

240. GREENBERG, L.J. and D. GLICK; J. Biol. Chem. 235: 3028, 1960.

241. MCKERNS, K.W.; Biochim. Biophys. Acta 71: 710, 1963.

242. OLDHAM, S.B., J.J. BELL and B.W. HARDING; Arch. Bioch.Biophys. 123: 496, 1968.

243. SAUER, L.A. and P.J. MULROW; Arch. Bioch. Biophys. 134: 486, 1969.

244. CAMMER, W. and R.W. ESTABROOK; Arch. Bioch. Biophys. 122: 721, 1967.

245. _____, ibid., 722 p.

246. PERON, F.G. and J.L. MCCARTHY; "Corticosteroidogenesis in Rat Adrenal Gland" in Functions of Adrenal Cortex. K.W. McKerns, ed. New York (Division of Meridith Corp.): Appleton-Century-Crofts, 1968. 285 p.

247. HAYANO, M., R.I. DORFMAN and E. ROSEMBERG; Fed. Proc. 14: 224, 1955.

248. SWEAT, M.L. and M.D. LIPSCOMB; Fed. Proc. 14: 290, 1955.

249. HAYANO, M. and R.I. DORFMAN; J. Biol. Chem. 201: 231, 1953.

250. PERON, F.G. and J.L. MCCARTHY; "Corticosteroidogenesis in Rat Adrenal Gland" in Functions of Adrenal Cortex. K.W. McKerns, ed. New York (Division of Meridith Corp.): Appleton-Century-Crofts, 1968. 264 p.

251. PERON, F.G., F. GUERRA and J.L. MCCARTHY; Biochim. et Biophys. Acta 110: 277, 1965.

252. LEVY, H., R.W. JEANLOZ, C.W. MARSHALL, R.P. JACOBSEN, O. HECHTER, V. SCHENKER and G. PINCUS; J. Biol. Chem. 203: 433, 1953.

253. BRODIE, B.B., J. AXELROD, J.R. COOPER, L. GAUDETTE, B.N. LADU, MITOME and S. UDENFRIEND; Science 121: 603, 1955.

254. GRANT, J.K. and A.C. BROWNIE; Biochim. et Biophys. Acta 18: 433, 1955.

255. GRANT, J.K. and K. MONGKOLKUL; Biochem. J. 71: 34, 1959.

256. BLOOM, B.M. (1962); Quoted by M. Hayano (1962) in "Oxygenase". O. Hayaishi, ed. New York: Academic Press, 181 p.

257. MASON, H.S.; Advances in Enzymology 19: 79, 1957.

258. COOPER, D.Y., R.W. ESTABROOK and O. ROSENTHAL; J. Biol. Chem. 238: 1320, 1963.

259. HAYANO, M., M.G. LINDBERG, R.I. DORFMAN, J.E.H. HANCOCK and W. von E. DOERING; Arch. Bioch. Biophys. 59: 529, 1957.

260. CAMMER, W. and R.W. ESTABROOK; Arch. Bioch. Biophys. 122: 735, 1967.

261. GARFINKLE, D.; Arch. Bioch. Biophys. 77: 493, 1958.

262. KLINGENBERG, M.; Arch. Bioch. Biophys. 75: 376, 1958.

263. ESTABROOK, R.W., D.Y. COOPER and O. ROSENTHAL; Bioch.Z. 338: 741, 1963.

264. OMURA, T. and R. SATO; J. Biol. Chem. 239: 2370, 1964.

265. HARDING, B.W., S.H. WONG and D.H. NELSON; Biochim. Biophys. Acta 92: 415, 1964.

266. NAKAMURA, Y. and H. OTSUKA; Biochim. Biophys. Acta 122: 34, 1966.

267. COOPER, D.Y., B. NOVACK, O. FOROFF, A. SLADE, E. SAUNDERS, S. NARASIMHULU and O. ROSENTHAL; Fed. Proc. 26: 341, Abst. 478, 1967.

268. SIMPSON, E.R., D.Y. COOPER and R.W. ESTABROOK; Rec. Prog. Horm. Res. 25: 528, 1969.

269. ROSENTHAL, O. and D.Y. COOPER; Methods of Enzymology. Vol. 10 New York: Academic Press, 1964, 616 pp.

270. PERON. F.G. and J.L. MCCARTHY; "Corticosteroidogenesis in Rat Adrenal Gland" in Functions of Adrenal Cortes. K. W. McKerns, ed. New York (Division of Meredith Corp.): Appleton-Century-Crofts, 1968. 286 p.

271. SIMPSON, E.R., D.Y. COOPER and R.W. ESTABROOK; Rec. Prog. Horm. Res. 25: 535, 1969.

272. _____, ibid., 552 p.

273. PURVIS, J., R. BATTU and F.G. PERON; "Functions of Adrenal Cortex," K. W. McKerns, ed. New York: Appleton-Century-Crofts, 1967.

274. OLDHAM, S.B., J.J. BELL and R.W. HARDING; Arch. Bioch. Biophys. 123: 506, 1968.

275. PERON, F.G.; Proc. Intern. Symp. Protein Polypeptide Hormones, Part 3. Liegé, Belgium, 1968. 164 p.

276. ALLEN, W.M.; J. Clin. Endoc. 10: 71, 1950.

277. CAMPBELL, W.R. and M.I. HANNA; J. Biol. Chem. 119: 1, 1937.

278. OKITA, G.I., J.J. KABARA, F. RICHARDSON and G.V. LEROY; Nucleonics 15: 111, 1957.

279. SIMPSON, S.A., J.F. TAIT, A. WETTSTEIN, R. NEHER, VON EUW J., O. SCHINDLER and T. REICHSTEIN; Helv. Chim. Acta 37: 1200, 1954.

280. QUESENBERRY, R.O. and F. UNGAR; Analyt. Biochem. 8: 192, 1964.

281. LISBOA, B.P.; Steroids 6: 605, 1965.

282. WEIST, W.G.; Steroids 10: 257, 1967.

283. BUSH, I.E.; Biochem. J. 50: 370, 1952.

284. SAFFRAN, M., J. BAYLISS and J. L. WEBB; Fed. Proc. 10: 116, 1951.

285. HALKERSTON, I.D.K., M. FEINSTEIN and O. HECHTER; Fed. Proc. 25: 494, 1966 (Abstract).

286. HALKERSTON, I.D.K., M. FEINSTEIN and O. HECHTER; Endocrinology 83: 61, 1968.

287. ROTHSTEIN, A. and R. MEIER; J. Cell. and Comp. Physiol. 34: 97, 1949.

288. ROTHSTEIN, A., R.C. MEIER and T.G. SCHARFF; Am. J. Phys. 41: 41, 1953.

289. LAPLANTE, C. and J. STACHENKO; Can. J. Biochem. 44: 85, 1966.

290. SIMPSON, E.R., D.Y. COOPER and R. W. ESTABROOK; Rec. Prog. Horm. Res. 25: 557, 1969.