OXYGEN FUNCTION IN CORTICOSTERONE BIOSYNTHESIS

by

Lawrence H. Heiselt, B. S.

A THESIS

presented to the Department of Biochemistry
and the Graduate Division
of the University of Oregon Medical School
in partial fulfillment
of the requirements for the degree of
Master of Science

June 1958

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

ACKNOWLEDGEMENTS

The author wishes to extend his thanks to Dr. Howard S. Mason, Dr. Robert A. Aldrich and Dr. Max L. Sweat for their help and guidance in the development of this project.

The author also thanks Dr. E. S. West for his constant encouragement and friendly interest.

The author is also indebted to C. H. deBruin for his assistance during the experimental work concerned with this thesis. Acknowledgement is also made to Drs. K. Yasunobu and W. L. Fowlks, who pyrolyzed the steroid samples.

l.r.h.

TABLE OF CONTENTS

CHAPTER		PAGE
I	INTRODUCTION.	1
	A. Experimental Development of Adrenal Steroid Biosynthetic Sequence.	1
	B. Reactions in Adrenal Steroid Biosynthesis	12
	No. 1. Breakdown of the Cholesterol Side Chain from C_{27} to C_{21}	12
	No. 2. Conversion of the Δ^{5}-3_{β} Hydroxy Group to the Δ^{4}-3 Ketone	13
	No. 3. Hydroxylation at 17α Position	14
	No. 4. Hydroxylation at the 21 Position.	16
	No. 5. Hydroxylation at the 11_{β} Position	17
II	MATERIALS AND METHODS	24
	A. Isolation and Preparation of Adrenal Mitochondria	24
	B. Incubation of Desoxycorticosterone With Adrenal Mitochondria.	25
	C. Crystallization.	37
	D. Identification of Biosynthesized Steroid.	37
	E. Analysis of Corticosterone for O^{18}.	40
III	RESULTS	42
	A. Incubation of Desoxycorticosterone With Adrenal Mitochondria.	42
	B. Characterization of Biosynthesized Steroid.	43
	C. Incorporation of O_2^{18} into Biosynthesized Corticosterone.	46
IV	DISCUSSION.	48
	A. Cofactor Requirements for 11_{β}-Hydroxylation in Various Tissue Preparations	49

TABLE OF CONTENTS (CONTINUED)

CHAPTER		PAGE
	B. Proposed Mechanisms of 11_{β}-Hydroxylation.	51
	Summary	55
BIBLIOGRAPHY.		56

LIST OF TABLES

TABLE		PAGE
I	Compounds Isolated After Perfusion of Various Steroids Through the Isolated Bovine Adrenal.	6
II	Steroid Transformations Achieved in Homogenates.	10
III	Separation of Corticosteroids on Silica Gel Column	36
IV	Separation and Determination of Steroids from Silica Gel Column	44
V	Mass Spectrometer Analysis for O_2^{18} . . .	47

LIST OF FIGURES

FIGURE		PAGE
1	Cholesterol Corticosteroidogenic Sequence	4
2	Silica Gel Chromatographic Column	31

CHAPTER I

INTRODUCTION

The subject of this thesis is the mechanism of enzymic 11_{β}-hydroxylation of desoxycorticosterone. This problem is of general interest for several reasons. It is concerned with the metabolism of steroid hormones, with the metabolism of oxygen and with the process by which enzymatic catalysis takes place. In describing the thesis work that has been done, and its significance, a review of steroid hormone biosynthesis will be presented first.

A. Experimental Development of Adrenal Steroid Biosynthetic Sequence

Studies of the biosynthesis of adrenocortical steroids began with the identification of approximately 30 steroidal substances by Kendall and Mason,(1) Wintersteiner(2) and Reichstein.(3) After the structural characterization of these compounds, the problem of the mechanism of their biosynthesis was then investigated.

Vogt(4) in 1943 used the direct method of adrenal venous blood sampling combined with bioassay techniques to evaluate the biological activity of the adrenal secretory products in the living animal. Following this study microchemical techniques were introduced which allowed the

isolation and identification of individual steroids in adrenal venous blood.(44) Extensive variations in the different steroid patterns have been found, with respect to relative proportions and individual components when different species were compared.(5) 17-Hydroxycorticosterone and corticosterone appear to be the predominant hormones found. Occasionally 11-dehydrocorticosterone is also found. A substance with high electrolyte regulating activity, presumably aldosterone(6) and other unidentified substances are normally secreted.

The discovery that the perfused adrenal gland can synthesize adrenocortical steroids from endogenous precursors, that this production can be significantly enhanced by the addition of ACTH to the perfusing medium and that a variety of individual biosynthetic reactions can be demonstrated by employing proper substrates was an important stimulus to the study of adrenocortical hormone biosynthesis.(7) The first demonstration that both acetate and cholesterol act as precursors was achieved in the isolated cow adrenal. Stone and Hechter(8) have studied the production of labeled 17-hydroxycorticosterone and corticosterone when C^{14}- labeled acetate, cholesterol and progesterone are perfused through the bovine adrenal stimulated with ACTH. The conversion of progesterone to these two adrenocortical steroids was not enhanced by

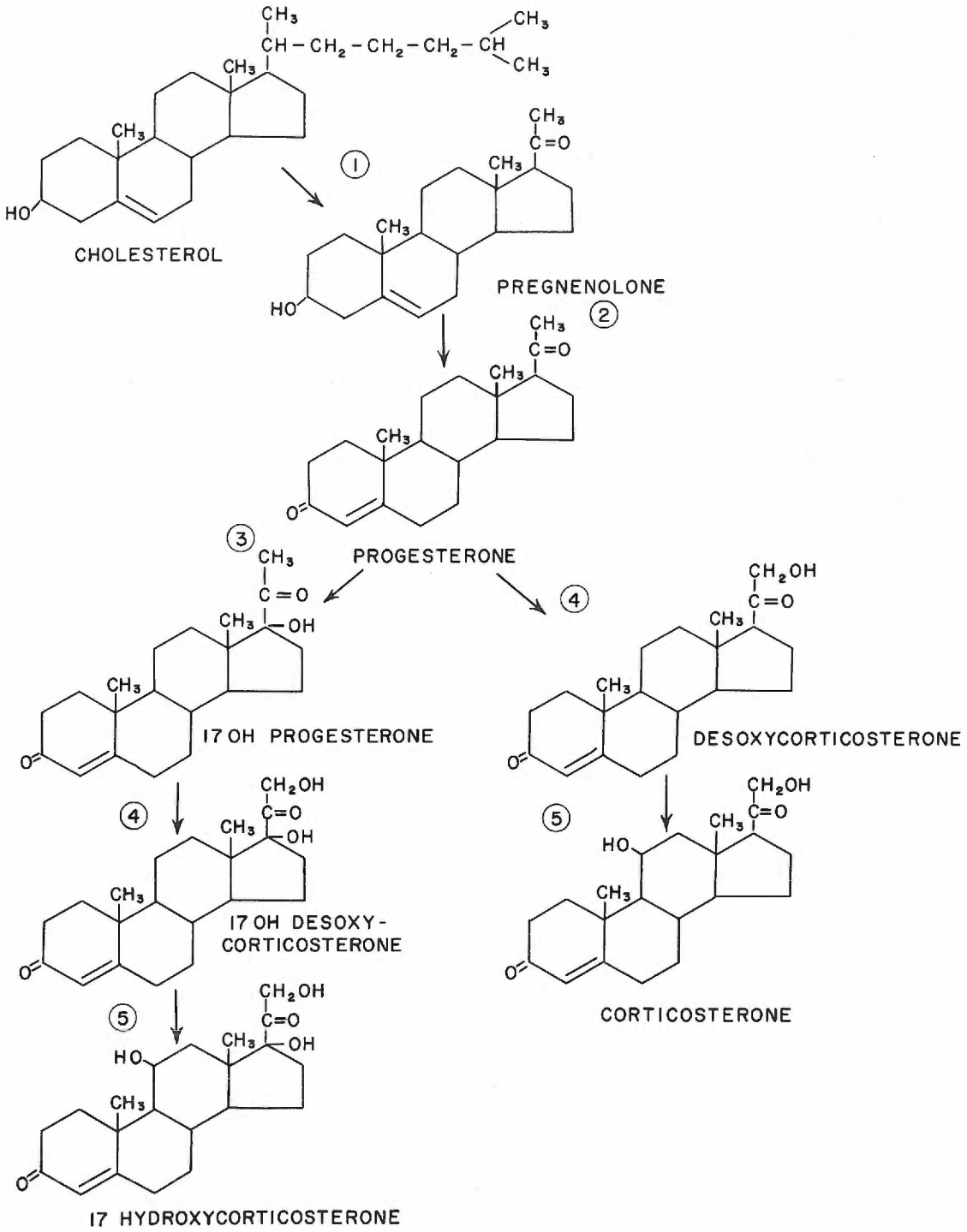
ACTH, whereas the synthesis of the corticoids was enhanced 18-fold by ACTH when C^{14} labeled cholesterol was employed. Acetate -1-C^{14} when perfused through a bovine adrenal was converted to 17 hydroxycorticosterone and corticosterone and this production was enhanced by ACTH about 1.4 - fold. It would appear that corticosteroids can be synthesized from cholesterol stored in the adrenal and that this pathway is under the stimulatory control of ACTH. They also may be synthesized by the alternative pathway of acetate conversion to corticosteroids, which does not involve acetate conversion to cholesterol and is not significantly increased by ACTH. Knowing that cholesterol is the major precursor and that hydrocortisone and corticosterone are major end products, it becomes possible to indicate the type of reactions which are necessary to achieve this transformation in adrenal tissue. These are depicted in Figure 1. From this Figure, it is evident that the following reactions must occur:

1. Breakdown of side chain from C_{27} to C_{20}.
2. Formation of an α - β unsaturated ketone in ring A.
3. Hydroxylation at C-17-α position.
4. Hydroxylation at C-21 position.
5. Hydroxylation at 11_{β} position.

Figure 1

Cholesterol Corticosteroidogenic Sequence

CHOLESTEROL CORTICOSTEROIDOGENIC SEQUENCE



Each of these reactions has been studied by Hechter, Pincus et al,(7,8,13,16,17,18) who utilized the isolated, perfused, adrenal gland. This method provides a system whereby cellular metabolism can be studied at a higher level of organization than in the tissue slice or homogenate. Since the best of perfusion conditions cannot exactly duplicate the physiological conditions which arise in vivo the perfusion method in common with other in vitro techniques can only reveal reactions which may occur in vivo but affords no guarantee that they are of physiological importance. They do lead to the development of concepts which may be tested experimentally in the living animal and at the cellular and sub cellular level in tissue slices and homogenates. The data collected by the above mentioned authors are summarized in the following table.

TABLE I

COMPOUNDS ISOLATED AFTER PERFUSION OF

VARIOUS STEROIDS THROUGH THE ISOLATED BOVINE ADRENAL

Compound Perfused	Products Isolated	Type of Reaction	Reference
cortisone	allopregnon-3$_\beta$-17α 21-triol-20-dione	Δ^4 saturation hydroxylation at C-3	13
	allopregnon-17α 21 diol-3, 11, 20-triones	Δ^4 saturation	
corticosterone	none		7, 14
17-hydroxycorticosterone	none		15
desoxycorticosterone	corticosterone	11$_\beta$ hydroxylation	4, 7
	allopregnon-21-ol-3, 20-dione	Δ^4 saturation	14
17-hydroxy-11-desoxycorticosterone	17-hydroxycorticosterone	21 hydroxylation 11$_\beta$ hydroxylation	16
21-desoxycortisone	cortisone	21 hydroxylation	17
	allopregnon-17α 21-diol-3, 11, 20-trione	Δ^4 saturation 21 hydroxylation	

TABLE I (CONTINUED)

Compound Perfused	Products Isolated	Type of Reaction	Reference
17-hydroxy-progesterone	17-hydroxycorticosterone	21 hydroxylation 11_β hydroxylation	7
	allopregnon-17_α -ol 3, 20-dione	Δ^4 saturation	14
	allopregnon-3_β, 17_α diol -20-one	Δ^4 saturation 3 hydrogenation	
Δ^4-pregnene-17_α 20, 21-triol-20-one	none		7
11_β-hydroxy-progesterone	none		14
progesterone	17-hydroxyprogesterone	17 hydroxylation	7, 14
	corticosterone	21 hydroxylation 11_β hydroxylation	
	17-hydroxycortico-sterone	17 hydroxylation 21 hydroxylation 11_β hydroxylation	
	11_β-hydroxyprogesterone	11_β hydroxylation	
	allo pregnon-3_β, 17_α diol-20-one	17 hydroxylation Δ^4 saturation 3 hydrogenation	
	allopregnon-17_α -ol 3, 20-dione	17 hydroxylation Δ^4 saturation	
	6_β-hydroxyprogesterone	6_β-hydroxylation	
	17-hydroxy-11-desoxy-corticosterone	17 hydroxylation 21 hydroxylation	

TABLE I (CONTINUED)

Compound Perfused	Products Isolated	Type of Reaction	Reference
pregnenolone	17-hydroxycorticosterone	3_β dehydrogenation Δ^4 dehydrogenation Δ^5 saturation 17 hydroxylation 21 hydroxylation 11_β hydroxylation	
	corticosterone	3_β dehydrogenation Δ^4 dehydrogenation Δ^5 saturation 21 hydroxylation 11_β hydroxylation	7
	progesterone	3_β dehydrogenation Δ^4 dehydrogenation Δ^5 saturation	
allopregnon-21-ol-3, 20-dione	allopregnon-11_β, 21-diol-3, 20-dione	11_β hydroxylation	
	allopregon-3_β, 21-diol-20-one	3_β hydrogenation	17
androstendione	Δ^4-androsten-11_β-ol-3, 17-dione	11_β hydroxylation	
	androstan-11_β-ol-3, 17-dione	11_β hydroxylation Δ^4 saturation	18
cholesterol	17-hydroxycorticosterone	Degradation of side chain; 3_β dehydrogenation; Δ^4 dehydrogenation; Δ^5 saturation; 17 hydroxylation; 21 hydroxylation; 11_β hydroxylation	
	corticosterone	Degradation of side chain; 3_β dehydrogenation; Δ^4 dehydrogenation; Δ^5 saturation; 21 hydroxylation; 11_β hydroxylation	9
	progesterone	Degradation of side chain; 3_β dehydrogenation; Δ^4 dehydrogenation; Δ^5 saturation	

Information about steroid transformations which has been obtained in experiments with bovine adrenal perfusions as summarized in Table I have been partially substantiated by the results of studies utilizing adrenal homogenates.

The conversion of Δ^5-3B OH sterols to $\Delta^4$3 ketones and the 17, 20 and 11$_\beta$-hydroxylations have been achieved with adrenal homogenates.

Table II provides a summary of the work which has been done with adrenal homogenates. It gives the compounds which have been used as substrates, the products which have been isolated and the type of tissue preparation.

TABLE II

STEROID TRANSFORMATIONS ACHIEVED IN HOMOGENATES

Compound Incubated	Homogenate Fraction	Products Involved	Reference
pregnenolone	whole	progesterone	19
dehydroepiandrosterone	whole	androstendione	19
progesterone	whole	desoxycorticosterone corticosterone 17-hydroxy-11-desoxycorticosterone hydrocortisone	11, 20
progesterone	supernatant	17-hydroxy-11-desoxycorticosterone desoxycorticosterone	21
17-hydroxyprogesterone	whole	hydrocortisone	11, 20
17-hydroxyprogesterone	supernatant	17-hydroxy-11-desoxycorticosterone	21
desoxycorticosterone	washed residue	corticosterone	11
17-hydroxy-11-desoxy-corticosterone	washed residue	hydrocortisone	11
androstenedione	washed residue	11β-hydroxyandrostenedione	11
pregnenolone	washed residue	no product	11
progesterone	washed residue	no product	11
17β-hydroxyprogesterone	washed residue	no product	11
androsterone	washed residue	no product	11

The following generalizations may be drawn from the results which are summarized in Tables I and II:

1. 11_β Hydroxylation is most efficiently accomplished when Δ^4-3 ketonic ketols are substrates.

2. It appears that hydroxylation at C-21 must precede 11_β-hydroxylation.

3. Since neither desoxycorticosterone or corticosterone is 17 hydroxylated either in adrenal perfusion or adrenal homogenates it is clear that the hydroxylation at C-17 must precede the hydroxylations at C-21 or C-11 to produce hydrocortisone.

4. Evidence suggests that when the 11_β hydroxyl function is present in the nucleus it is difficult to introduce the 17 or 21 hydroxyl function. Thus perfusion of 11_β hydroxyprogesterone does not yield hydrocortisone or corticosterone in signficant amount nor are significant amounts of ketol formed. Similarly corticosterone is not converted to hydrocortisone.

5. If instead of 11_β hydroxyl one introduces the 11α hydroxyl grouping the situation is completely changed. 11α Hydroxyprogesterone is readily hydroxylated at C-17 and C-21 by adrenal homogenates.(10) The keto grouping is similar to the 11α hydroxyl grouping in that 21 hydroxylation readily occurs both in perfusion and homogenates.(11)

B. Reactions in Adrenal Steroid Biosynthesis

With these generalizations in mind we may discuss in more detail the individual steps which occur during the biosynthesis of the adrenal cortical steroids, corticosterone and 17-hydroxycorticosterone, from cholesterol. These steps will be taken up in the order in which they are given in Figure1.

No. 1. Breakdown of the Cholesterol Side Chain from C_{27} to C_{21}

Little is known of reaction No. 1 that takes place between cholesterol and the first postulated intermediate pregnenolone.(25) Some information may be gained from the fact that when C^{14} cholestenone is perfused through glands for three hours with and without added ACTH, although sizable amounts of corticosteroids are produced with ACTH, the 17-hydroxycorticosterone and corticosterone spots eluted following paper chromatography do not contain measurable radioactivity.(12) This would appear to exclude cholestenone from the pathway and suggests that the removal of the side chain (C_{27}--C_{21}) precedes the subsequent oxidation at C-3 to form the Δ^4-3 ketosteroid grouping.

No. 2. Conversion of the Δ^5-3_β Hydroxy Group to the Δ^4-3 Ketone

Reaction No. 2 deals with the action of a steroid 3B ol dehydrogenase which is present in abundant concentration in the adrenal cortex. This enzyme oxidizes the Δ^5-3B hydroxy groups of both pregnenolone and dehydroepiandrosterone to the corresponding Δ^4-3 ketosteroids namely progesterone and Δ^4 androstene 3,17-dione. The steroid 3_β ol dehydrogenase has been found to be associated with the microsomal fraction of adrenal homogenates,(22) requires DPN* as a cofactor,(23) and is a lipoprotein in basic structure.(22) This type of enzymic activity, the conversion of Δ^5-3_β hydroxy compounds into Δ^4-3 ketosteroids, is not confined to the adrenal cortex but also occurs in the corpus luteum, placenta, and the interstitial cells of the testis. Some of the structural characteristics of the steroids oxidized by this dehydrogenase and their influence on rates of reactions have been studied by Samuels.(24) It was found that the compounds having a 3_β hydroxy group are metabolized much more

---

*Abbreviations that will be used in this thesis are as follows: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

rapidly than their 3α isomers. The presence or absence of the double bond seemed to make little difference in the oxidative rate but an alcohol or ketone group in position 17 or 20 markedly increased the rate of oxidation. The presence of a methyl group on carbon 17 decreased the rate of reaction.

No. 3. Hydroxylation at 17α Position

Reaction No. 3, the conversion of progesterone to 17α - hydroxyprogesterone, has been demonstrated by perfusion studies(26) and studies using beef adrenal homogenates.(26,27) Plager and Samuels working with the supernatant fraction of beef adrenal homogenates centrifuged at 20,000xg for 0.5 hour reported the presence of enzyme systems which will oxidize the 21-methyl group of progesterone to a primary alcohol group and introduce a 17-hydroxyl group into the progesterone molecule.(20) On investigating the cofactor requirements for the 17 hydroxylating system it was found that neither ATP or DPN alone could introduce the hydroxyl group into the steroid nucleus but required the presence of both substances.(20,21) The authors offered the following explanation:

> Since high energy phosphate is required for the reaction and examples of the utilization of this type of energy have so far been usually found to involve the formation of a phosphate intermediate with the substrate it

> is not unlikely that a similar phosphate intermediate is essential for the conversion of progesterone to 17-hydroxyprogesterone.[20]

This type of compound is one possible explanation of a "water soluble loss" noted in incubations in which high 17-hydroxylating activity was obtained.[26] The best alternative to this interpretation of the data would require this enzyme system to have a specificity for TPN and that the DPN and ATP utilized as cofactors are acting in the synthesis of TPN.

From information gained from adrenal perfusion studies Hechter et al[7,12] have proposed that 17-hydroxylation cannot occur after 21-hydroxylation in the biological formation of 17-hydroxycorticosterone. They noted that progesterone was converted to corticosterone and 17-hydroxycorticosterone while desoxycorticosterone went only to corticosterone. Since this sequence of steroid biosynthesis could have resulted from some characteristic of their[7,12] procedure rather than from enzymatic specificity of the 17-hydroxylating system, it was checked by Plager[26] utilizing the supernatant fraction of adrenal homogenates.

His data confirmed the order of 17- and 21-hydroxylation as proposed by Hechter. The results can be depicted as follows:

SUBSTRATE	PRODUCT
1. Progesterone	17-hydroxy-11-desoxycorticosterone 25%
	11-desoxycorticosterone 55%
2. 17-hydroxyprogesterone	17-hydroxy-11-desoxycorticosterone 60%
	11-desoxycorticosterone none
3. 11-desoxycorticosterone	17-hydroxy-11-desoxycorticosterone none

There was also found to be a sex difference in enzymatic activity, the adrenals from females producing a greater amount of 17-hydroxy-11-desoxycorticosterone. The production of this compound by bull adrenals was less and that of steer adrenals least.(26)

No. 4. Hydroxylation at the 21 Position

Reaction No. 4, 21 hydroxylation, is catalyzed by the same cellular fraction which carries out 17 hydroxylation, the supernatant fraction of adrenal homogenates obtained by centrifugation. Other characteristics of the 21-hydroxylase system are similar to the 17 hydroxylase system. It has been found that the most marked amounts of 21-oxidation take place in the presence of DPN plus ATP but there is significant formation of desoxycorticosterone when progesterone is incubated with DPN alone. This might indicate that the reaction proceeds with DPN as hydrogen

transferring agent but if enough ATP is present TPN may be formed and the reaction proceeds with an increased rate of 21-oxidation. It was observed by Samuels and Plager[27] that the addition of dehydroisoandrosterone to the medium seems to affect the relative yields of 11-desoxycorticosterone and 17-hydroxy-11-desoxycorticosterone from incubated progesterone.

No. 5. Hydroxylation at the 11_β Position

Reaction No. 5, 11_β-hydroxylation, takes place in every major pathway of adrenal cortical steroid biosynthesis and has been referred to as the reaction that gives the adrenal steroid molecule its trade mark of a finished product.[19] In 1949 Hayano et al[20] incubated desoxycorticosterone with adrenocortical slices and adrenal homogenates and demonstrated the formation of material which possessed glycogenic activity when tested in the fasting adrenalectomized mouse. The conclusion was reached that 11_β-hydroxylation had occurred and that desoxycorticosterone was converted to corticosterone. This enzyme system has been studied in some detail. Only the experimental development will be taken up here. The theoretical considerations will be reserved for later discussion.

In 1950 McGinty,(29) utilizing a whole adrenal homogenate supplemented with glucose and fumarate, isolated 17α-hydroxycorticosterone as a transformation product of 17α-hydroxy-11-desoxycorticosterone. Savard, Green and Lewis(30) obtained evidence for formation of 11$_\beta$-hydroxyderivates from 11-desoxycorticosterone on incubation with adrenal homogenates without added fumarate or other cofactors.

Kahnt and Wettstein(31) confirmed the stimulatory effect of added fumarate and obtained evidence that ATP could replace fumarate as a cofactor. They also stated that nicotinamide and fumarate stimulated the preparation maximally. Glucose, inorganic phosphate and magnesium ions were without effect. These authors also suggested that the process of oxidative phosphorylation may be involved.

Sweat(32) demonstrated that 11$_\beta$-hydroxylating activity is associated with the mitochondrial fraction of adrenal homogenates. On the basis of heat, rate, and inhibitor studies concluded that the conversion was enzymic in nature. These studies were done utilizing 17α-hydroxy-11-desoxycorticosterone as substrate, incubating it with adrenal cell particles in a medium supplemented with glucose, fumarate and magnesium ions.

Hayano and Dorfman[33] found that if homogenate residues by centrifugation at 5000 X gravity, are washed with saline phosphate buffer, the washed residues are no longer effective and restoration of 11_{β}-hydroxylation activity requires the addition of fumarate and Mg++. Upon further study Hayano showed that washed residues prepared from glands stored at 0° for long periods required ATP, DPN and Mg++ plus fumarate or TPN plus fumarate in order to restore activity.[33]

In 1954 Brownie and Grant,[34] utilizing intact adrenal mitochondria, studied the effectiveness of Krebs cycle intermediates as activators of 11_{β}-hydroxylation of 11-desoxycorticosterone. Their results indicated that fumarate, malate, succinate, citrate, oxalacetate, and α ketoglutarate all catalyze the reaction; α ketoglutarate had the most marked effect. These experiments do not eliminate the possibility of some special requirement for fumarate since this substance will be formed from other intermediates by operation of the Krebs cycle. The authors used the inhibition of succinate by malonate to show that the one step reaction α ketoglutarate to succinate could activate 11_{β}-hydroxylation of 11-desoxycorticosterone. These results indicate that there is no specific requirement for fumarate in intact mitochondria. It was also noted by these investigators

that dinitrophenol, 2 x 10^{-4}M, gave a 50 per cent inhibition of 11_{β}-hydroxylation. Sweat and Lipscomb(35) demonstrated that the glucose-6-phosphate-TPN system was more effective in activating 11_{β}-hydoxylation of desoxycorticosterone than either DPNH or TPNH. Additional evidence for the requirement of TPNH was obtained by a study of the rates at which citrate, cis-aconitate and isocitrate in the presence of TPN enhance the rate of desoxycorticosterone hydroxylation. Their data show that isocitrate was the most effective of the three acids. It appears that in the 11_{β}-hydroxylation of 11-desoxycorticosterone the effects of the di- and tricarboxylic acids are largely due to their role in supplying TPNH. Consistent with this conclusion are other results reported by these investigators that potassium ferricyanate, methylene blue, toluidine blue, azure I, methyl viologen and benzyl viologen all of which are effective electron acceptors, markedly inhibit the reaction.

More recent studies by Grant and Brownie(36) and Hayano, Dorfman and Rosenberg(37) reveal the fact that water-soluble extracts of acetone dried powders from adrenal mitochondrial preparations possess the ability to introduce a 11_{β}-hydroxyl group into 11-desoxy corticosterone.

The enzymic activity of these clear red water soluble extracts obtained by centrifuging at 20,000xg for one-half hour have the following characteristics:

1. Hydroxylation does not occur in absence of oxygen.
2. Hydroxylation is inhibited by phosphate ions.
3. Hydroxylation is inhibited by dialysis against distilled water.
4. Hydroxylation is slightly inhibited by 10mMKCN.

It was also noted that fumarate could be replaced by L-malate or isocitrate but not by α ketoglutarate, the most potent activator of 11_{β}-hydroxylation with intact mitochondria.

Previous speculation on the mechanism of the introduction of a hydroxyl group into the $C11_{\beta}$ position of a steroid was based on an analogous situation, the well known step-wise transformation of succinate -- fumarate -- malate involving the obligatory unsaturated derivatives. Desoxycorticosterone was visualized to proceed primarily to the Δ 9(11) or the Δ 11(12) compound which would then be hydroxylated by the asymmetric addition of the elements of water to yield the product corticosterone.

This question was studied by Heyano and Dorfman.(38) Investigation of the Δ 9(11) unsaturated analogues of desoxycorticosterone and 11-desoxycortisol as possible

intermediates in the conversion of desoxycorticosterone to corticosterone and 11-desoxycortisol to cortisol demonstrated no utilization of these structures. When carried out in a deuterium oxide containing media no deuterium entered a stable position on the molecule, in the course of 11_{β}-hydroxylation. These facts would exclude the possibility of any biosynthetic mechanism involving the direct addition of water.

In summary, 11_{β}-hydroxylation of desoxycorticosterone has been demonstrated in the whole homogenates of adrenal glands, the intact adrenal mitochondria, disrupted adrenal mitochondria, acetone powders and aqueous extracts of acetone powders. The cofactor requirements depend upon the tissue preparation. Intact mitochondria require oxygen. Various members of the Krebs cycle, TPN and Mg^{++} are stimulatory. Disrupted mitochondria require oxygen, fumarate, Mg^{++}, at PH 7.4 and added TPN stimulates the reaction.

When acetone powder extracts were used oxygen and TPN were required and fumarate had the greatest stimulatory action. 11_{β}-Hydroxylation has been most effectively inhibited by compounds that act as electron acceptors such as methylene blue. Other substances inhibiting this reaction are ascorbic acid, glutathione, HCN, diethylithiocarbamate, methylene blue, arsenate, fluoride, versene, and

desoxycorticosterone.

Studies of the mechanism of 11_{β}-hydroxylation excluded intermediate double bonded structure and the possibility of a hydroxylation mechanism involving the direct hydration of an intermediate structure. Further studies on the mechanism of 11_{β}-hydroxylation and more specifically the source of oxygen for this hydroxylation form the basis of the work to be presented in this thesis.

Recently Mason(39) has utilized $O_2{}^{18}$ and H_2O^{18} as tracers to determine the source of oxygen used by the phenolase complex in its hydroxylative phase. Similar methods to those used by Mason were utilized in the experiments that follow to determine the source of oxygen for the 11_{β}-hydroxylation of desoxycorticosterone.

CHAPTER II

METHODS AND MATERIALS

A. Isolation and Preparation of Adrenal Mitochondria

Adrenal glands obtained from Armour and Co. were stored in a deep freeze at -40°C for approximately one week. The glands were taken from the deep freeze to a cold room where they were allowed to thaw at a temperature of 0-4°C.

The following operations were performed at a temperature of 0-4°C. The glands were stripped of fat and connective tissue, then ground in a meat grinder which had been previously cooled. One hundred to one hundred and fifty grams of mince obtained by grinding whole glands were diluted with 500-750 ml of cold distilled water. Small aliquots of this suspension were transferred to a Waring Blender and homogenized for seven seconds. The homogenate obtained in this manner was strained through two layers of cheesecloth. The filtrate obtained by straining through cheesecloth was centrifuged in a refrigerated Servall centrifuge for five minutes at 300 x gravity. The supernatant was carefully syphoned off without disturbing the residue. The residue was discarded and the supernatant was placed in lucite tubes and

centrifuged in a refrigerated Servall centrifuge for 20 minutes at 17,000 to 20,000 x gravity. The supernatant fraction was siphoned from the mitochrondrial pellet together with as much of the yellow fat as possible. The tip of the siphon tube was placed near the surface of the supernatant fluid in order to remove the layer of yellow fat before it settled on the mitochondria. The inside walls of the tubes were wiped with an absorbent paper or cotton to remove the remaining fat.

The mitochondrial paste was transferred directly to the lower part of a Potter-Elvehjem glass homoginizer, diluted with 40-72 ml of distilled water and homogenized. This mitochondrial solution was then introduced into the incubation media.

B. Incubation of Desoxycorticosterone with Adrenal Mitochondria

The solutions that made up the incubation media were prepared as follows:

1. Reagents

a. Phosphate buffer

Solution A: 0.4M NaH_2PO_4
5.56 gm NaH_2PO_4 per 100 ml

Solution B: 0.4M Na_2HPO_4
5.69 gm Na_2HPO_4 per 100 ml

The phosphate buffer (pH7.4) used in the incubation media was a solution made by

adding 9.5 ml of A to 40.5 ml of B and diluting this up to 200 ml with distilled water.

b. Magnesium chloride Solution 0.04M

813.3 mg of $MgCl\text{-}6H_2O$ in 100 ml distilled water.

c. 0.1M Fumarate

11.8 gm of fumaric acid was placed in a one liter beaker surrounded by ice. Five hundred ml of distilled water was added and 9.5 ml of 10 N sodium hydroxide was then added drop by drop. Three to four drops of phenol red were then added and the solution was titrated with 1N sodium hydroxide until a red-orange end point was reached. The solution was transferred to a 1000 ml flask, diluted to the mark and stored at $4^{\circ}C$.

d. .0025 M TPN

The trisodium salt of tri phosphopyridine nucleotide obtained from Pabst Laboratories was used in preparing this solution. A stock solution with concentration of 84 mg per 10 ml of distilled water was made up and stored in the deep freeze. Immediately before making up the incubation mixture the stock solution

was thawed and 3 ml of stock solution was diluted up to 12 ml with distilled water to make a 0.0025M TPN solution.

e. Stock Solution of Desoxycorticosterone.

160 mg of desoxycorticosterone dissolved in 100 ml of absolute ethyl alcohol.

2. Preparation of Incubation Medium and Incubation Conditions:

The incubation medium or media were prepared as follows:

Eighteen ml. of the stock solution of desoxycorticosterone was transferred to the reaction flask and evaporated to dryness in an air stream. One ml of ethanol was then added and rotated until the desoxycorticosterone was completely dissolved. Twelve milliliters of each of the following solutions were added to the reaction flask:

(1) Phosphate Buffer PH 7.4,

(2) 0.04 M magnesium chloride,

(3) 0.1 M fumarate, and

(4) 0.0025M TPN.

Oxygen-free hydrogen gas was then bubbled through this reaction mixture for approximately 20 minutes. This procedure was done in

a partially closed system. The stop cock was then turned and a stream of oxygen generated from the attached electrolysis apparatus utilizing water enriched with O^{18} was bubbled through the incubation mixture.

After sweeping the mixture with oxygen enriched with $O_2{}^{18}$ the mitochondrial preparation was introduced into the reaction flask through a closed system after having hydrogen gas bubbled through it. This reaction mixture was agitated by the use of teflon covered magnetic stirring bar. Temperature was maintained by a water bath that surrounded the flask keeping the contents at a constant temperature of 35°C. This incubation of desoxycorticosterone with mitochondria in an $O_2{}^{18}$ enriched atmosphere continued for three hours.

3. Extraction of Incubation Media:

At the end of the three hour incubation period the reaction flask was removed from the oxygen generating apparatus and 200 ml of redistilled chloroform was introduced. This mixture of chloroform and incubation reactants were agitated by hand for about 10 minutes and then divided into

four equal parts, placed in 250 ml centrifuge tubes and centrifuged at 2500 r.p.m. for 10 minutes. The chloroform fraction was aspirated off and placed in a 1000 ml Erlenmeyer flask. Another 200 ml of chloroform was used to extract the incubation mixture a second and third time and the above procedure was repeated. The three 200 ml chloroform washes were combined in the 1000 ml Erlenmeyer flask and evaporated to dryness in an air stream at a temperature ranging from 45-50°C. This flask containing the residue of the chloroform extraction was usually stored in the refrigerator at 4°C until further purification could be carried out.

4. Purification of Biosynthesized Steroid

a. Partition Extraction

To the dry residue of the chloroform extraction was added 100 ml of hexane then 20 ml of 70 per cent ethanol. The flask was shaken and allowed to stand 30 minutes. The two liquids were again intermixed several times by shaking and transferred to a separatory funnel where they were allowed to separate. The 70 per cent ethanol layer was returned to the original flask and the hexane

was added to the flask and the process of shaking and transferring to the separatory funnel was repeated. Additions of 4 to 5 ml of 70 per cent ethanol were used to wash the original flask during each transfer. After the separatory funnel had been shaken for 30 seconds the ethanol layer containing the synthesized corticosterone was removed to round botton flask where it was evaporated to dryness at a temperature not exceeding 50°C. To this dried residue was added 5 ml of redistilled chloroform which was allowed to stand in a stoppered flask at room temperature for 30 minutes in preparation for chromatography.

b. Silica Gel Chromatography

The methods used in the purification of the above dried residue were a modification of the column chromatography techniques of Sweat.(40)

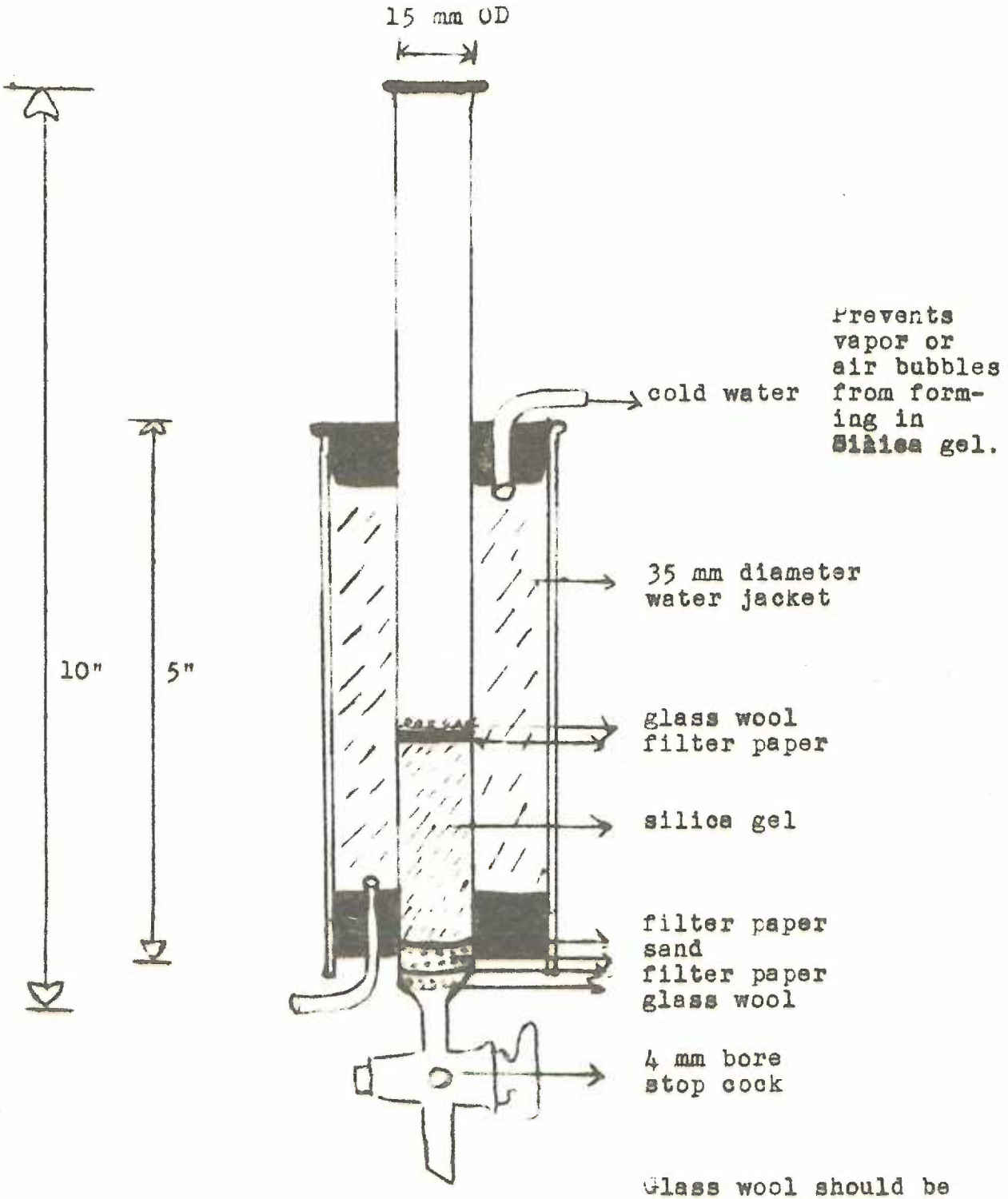
(1) Apparatus:

The chromatographic column employed is illustrated in Figure 2. Water jackets are necessary to keep the absorbent cool and prevent air bubble formation:

Figure 2

Silica Gel Chromatography Column

COLUMN FOR SEPARATING MILLIGRAM QUANTITIES OF STEROIDS



Glass wool should be cleaned in conc. HNO_3

Filter paper should be extracted with ETOH

Sand should be washed with ETOH and water.

(2) Reagents

Adsorbent:

A mixture of 250 ml of 95 per cent ethyl alcohol, 750 ml of chloroform and 200 grams of silica gel (Davison Chemical Corp., Baltimore, Md., silica gel No. 922-08-08-226-T200) was allowed to stand several hours. The solvent mixture was filtered under gentle suction on a Buchner funnel. The silica gel was washed successively with 400 ml of 3 to 1 ethyl alcohol-chloroform and 1 liter of 95 per cent ethyl alcohol. The partially dried silica gel was then transferred to a four liter beaker containing two liters of distilled water. Black particles were removed during the water washings by swirling the water silica gel mixture in suitable size beakers and decanting the suspended silica gel from the black particles which accumulated in the botton and center of the beakers. The silica gel was partially dried on a Buchner funnel and then spread on a clean surface to complete drying. After the silica gel

was completely dry and free of alcohol it was activated in a muffle furnace at 500°C for three hours.

Ethyl Alcohol:

Absolute ethyl alcohol was distilled from a water-free system; the first and the last 10 per cent of the distillate were discarded. The distilled ethyl alcohol was stored in the dark in bottles with close fitting glass stoppers.

Chloroform:

Mallickrodt analytical reagent grade chloroform supplied in glass containers was distilled from an all glass, water free distillation apparatus. The chloroform employed in chromatography represented the first 90 per cent of the distillate.

(3) Preparation of Chromatography Column

First a plug of washed glass wool was placed in the column then a small disc of filter paper was placed on top of the glass wool. With the stop cock open and with cold tap water running through the cooling jacket enough

chloroform washed sand was added to the column to form a layer 1 cm to 1.5 cm above the filter paper disc. The sand was washed into place with a small quantity of chloroform. The chloroform was allowed to drain; the stop cock was closed and another filter paper disc was placed on the top of the sand. A suspension of 2 to 3 grams of activated silica gel in 20 ml of redistilled chloroform was poured into the column. Two additional 5 ml quantities of chloroform were used to rinse the beaker and the sides of the column. The stop cock remained closed until the silica gel had settled completely then another filter paper disc and a glass wool plug were placed on top of the silica gel.

The chloroform was allowed to run off until its upper level coincided with filter paper on top of the silica gel. Three 10 ml quantities of chloroform were added to the column in succession and allowed to drain to the top of the silica gel.

(4) Addition of Sample and Development of Column

The 5 ml of chloroform containing the partially purified residue was transferred to the column and an additional 2-3 ml of chloroform was used to wash the container. When the upper surface of the chloroform solution reached the upper surface of the silica gel 20 ml of 1 per cent ethyl alcohol in chloroform was added to the column and the effluents from this fraction were collected in four 50 ml round bottom flasks. Four drops of the chloroform effluent was collected from each fraction in a small test tube. The chloroform was evaporated off and the residue was taken up in .1 ml ethanol and 2 ml of concentrated sulfuric acid was added. Each one of these samples were tested for the green fluorescence characteristic of corticosterone.(42) Additional chloroform and ethyl alcohol eluents were added to the column collected in 50 ml round bottomed flasks and analyzed as listed in the following table.

TABLE III

SEPARATION OF CORTICOSTEROIDS ON SILICA GEL COLUMN

	Vol. Eluent ml.	% Ethanol in Chloroform	Fraction Number	Steroid Resolved	Method Analysis
Cleaning of Column	10	0.0	A		
	10	0.0	B		
	10	0.0	C		
Addition of Steroid	5	0.0			
	5	1.0	A		
	5	1.0	B		
	5	1.0	C	Desoxycorticosterone	Sulfuric Acid Chromogens (41)
	5	1.0	D	17-OH-11-Desoxycorticosterone	
	5	5.0	E		
	5	5.0	F		
	5	5.0	G	Corticosterone	Sulfuric Acid Chromogens (41) Fluorescence (42)
	5	5.0	H		
	5	7.0	I	17-OH-Corticosterone	
	5	7.0	J		

Each one of the ethyl alcohol-chloroform effluents (A-J) were evaporated in vacuo at 50°C. The dried crystalline residues obtained by the above methods of purification were stored in refrigerator until analysis could be carried out. The results of the column chromatographic purification method will be discussed in the next section.

C. Crystallization

Further purification of fraction G and H from benzene was done as follows: 12 drops of benzene were added to the white residue, shaken, heated on a water bath at 40° and cooled rapidly in a bath of chopped ice. The mother liquor containing colored impurities was removed from the resulting white crystals by using a small filter stick. The tube containing the crystalline residue was sealed for use in the identification procedures which follow.

D. Identification of Biosynthesized Steroid

1. Fluorescence:

Of the relatively large number of corticosteroids tested by Reichstein and Shoppe[3] and by Wintersteiner and Pfiffner[43] only three substances corticosterone, 17-hydroxycorticosterone, and Δ^4-pregnene-11_β 17α 20 β 21-tetrol-3-one were found to exhibit sulfuric acid-induced fluorescence. The fluorescent properties of

corticosterone have been studied extensively by Sweat.(42) He has found that the maximal degree of fluorescence of corticosterone is induced by light of wave length in the 455 to 460 mu region and that the emission spectrum of the fluorescence produced by corticosterone in ethanolic sulfuric acid solution has a maximum at the 530 to 540 mu region. The small samples of 4 or 5 drops collected from the effluents of the silica gel column were examined for this characteristic fluorescence of corticosterone. This fluorescent property of corticosterone was one of the criteria used to indicate conversion of desoxycorticosterone to corticosterone.

2. Ultraviolet Spectrum:

Another method used for determining the activity of the incubation reaction and therefore the degree of synthesis of corticosterone was the measurement of the ultraviolet absorption of a solution of the purified steroid at 230, 240 and 260 mu. Steroids possessing a Δ^4-3 ketone group exhibit a strong absorption at 238 to 242 mu. The dried residue of the 10 effluents from the silica gel column were analyzed by dissolving them in 5 ml of absolute ethanol, transferring them to silica cuvettes and determining optical densities

in a Beckman D.U. spectrophotometer at the previously mentioned wavelengths. It was found that the maximum absorption at 240 mu was displayed by fractions C, D and G, H. This would correspond with Sweat's data[40] for elution of desoxycorticosterone and corticosterone.

3. Absorption Spectra of Sulfuric Acid Chromogens

The method used was that of Zaffaroni[44] in which 70 to 90 micrograms of dry steroid was placed in a tube and 3 ml concentrated sulfuric acid was added. The tube was stoppered and allowed to stand at room temperature for two hours. The absorption spectrum of this steroid sulfuric acid solution was obtained with the use of a Beckman D.U. spectrophotometer between the wavelengths 220 to 600 mu, using concentrated sulfuric acid as a blank. The following results were obtained by Zaffaroni et al:[44]

Steroid	Absorption Maximum
17 hydroxy corticosterone	280, 395, 475
Corticosterone	285, 330, 373, 455
Desoxycorticosterone	285, 370, 440

Following the above method a 1.0 mg sample was obtained from the combined dry residues from fractions G and H when biosynthesized corticosterone was separated on the silica gel column.

This sample was placed in a graduated cylinder and 30 ml of concentrated sulfuric acid was added. The solution was allowed to stand at room temperature for two hours and 0.5 ml aliquot was then read in a Beckman D.U. spectrophotometer between the wavelengths 220 and 660 mu. An authentic 1 mg sample of corticosterone was weighed and analyzed in the same manner.

4. Infrared Analysis of Biosynthetic Steroids

A 5 mg sample of the 75 mg of purified biosynthesized corticosterone was dried and sent to Oregon State College for infrared analysis. A 20 mg sample of purified corticosterone obtained from Merck Pharmaceutical Company was also analyzed as a control. A Nujol blank was used and the samples were analyzed in a Perkin-Elmer model 12-C modified single beam double pass infrared spectrometer using an NaCl prism.

E. Analysis of Corticosterone for O^{18}

1. Preparation:

The biosynthesized corticosterone purified by the previously mentioned techniques of partition extraction, column chromatography and recrystallization was transferred to small platinum boats for pyrolysis in the following manner. The 67 mg of

purified steroid material was divided into three parts by directly transferring the sample to the platinum pyrolysis boats by the use of a clean spatula. Care was taken not to introduce any foreign material into the sample that would alter the final results by introducing extraneous oxygen. The biosynthesized corticosterone and the boats were placed in a dissicator under reduced pressure for 24 hours and then pyrolyzed in an Unterzaucher pyrolysis apparatus.(45) The CO_2 formed from the pyrolyzed corticosterone was trapped in a thin evacuated glass tube and the tube was sealed. The CO_2 samples were analysed by mass spectrometry to determine the atoms per cent excess of O_2^{18} present in the CO_2 collected from the Unterzaucher pyrolysis procedure.

The mass spectrometry was carried out by the Consolidated Electrodynamics Corporation, Pasadena, California.

CHAPTER III

RESULTS

A. Incubation of Desoxycorticosterone with Adrenal Mitochondria

When the previously described procedures for isolation and incubation were used, the adrenal mitochrondria consistently had 11_{β}-hydroxylating activity except on one occasion, when the refrigerating mechanism of the deep freeze failed to maintain a -40°C temperature. The adrenal tissue that was stored in the deep freeze during this period of time (2-3 days) was later found to be inactive as far as 11_{β} hydrolating activity was concerned. This tissue was discarded and adrenal glands for subsequent experiments were obtained from the local Swift and Co. slaughter house. The preliminary tests for 11_{β}-hydroxylating activity were the appearance of a white crystalline residue upon evaporation of the solvent from fractions G and H collected from the silica gel column and the characteristic sulfuric acid induced fluorescence of corticosterone found in a aliquot of the incubation medium. The percentage conversion of desoxycorticosterone to corticosterone in a study by C. H. deBruin[47] utilizing the same incubation system was found to be 76.5 per cent.

B. Characterization of Biosynthesized Steroid

1. Resolution of steroids from silica gel column and measurement by fluorescence and absorption at 240 mu:

The separatory capacity of the silica gel column was tested by adding known amounts of desoxycorticosterone and corticosterone to the column and comparing the elution pattern with that of the biosynthesized steroid. The presence of corticosterone in the effluents was detected by the green fluorescence test for corticosterone and ultraviolet absorption at 240 mu as measured in the Beckman D.U. spectrophotometer.

TABLE IV

SEPARATION AND DETERMINATION

OF STEROIDS FROM SILICA GEL COLUMN

	Vol. Eluent ml.	% Ethanol in $CHCl_3$	Fraction Number	Corticosterone Desoxycorticosterone Fluorescence	Corticosterone Desoxycorticosterone O.D. 240	Biosynthesized Steroid Fluorescence	Biosynthesized Steroid O.D. 240
Cleaning of Column	10	0.0	A	0	.012	0	.010
	10	0.0	B	0	.029	0	.035
	10	0.0	C	+	.015	+	.033
Addition of Steroid	5	0.0		0	.011	0	.020
Development of Column	5	1.0	A	0	.011	+	.064
	5	1.0	B	+	.052	+	.042
	5	1.0	C	+	.267	+	.448
	5	1.0	D	0	.483	++	.450
	5	5.0	E	+	.086	+	.061
	5	5.0	F	+	.030	+	.068
	5	5.0	G	++++	1.130	++++	1.230
	5	5.0	H	++++	1.230	++++	1.670
	5	7.0	I	+	.031	+	.260
	5	7.0	J	+	.026	+	.062

2. Absorption Spectra of Sulfuric Acid Chromogens:

Authentic samples of corticosterone, desoxycorticosterone and the residue from effluents G and H were analyzed by the method of Zaffaroni[44] as described previously. The results obtained were as follows:

Steroids	Absorption Maxima
Authentic Corticosterone	285, 330, 375, 450
Desoxycorticosterone	290, 370, 440
Biosynthesized Steroid	285, 330, 375, 450

The absorption maxima of the biosynthesized steroid were the same as the standard corticosterone.

3. Infrared Spectra:

The infrared absorption spectra of the 5 mg sample of the 75 mg of pooled biosynthesized steroid and the sample of authentic Merck corticosterone showed that the absorption bands in the region between 1500 cm^{-1} and 1800 cm^{-1} were identical. The maximal absorption bands for the two samples were at 1700 cm^{-1} 1660 cm^{-1} 1645 cm^{-1}. This data coincides with the maximal absorption bands for corticosterone published by Dobriner.[46]

C. Incorporation of O_2^{18} into Biosynthesized Corticosterone

The expected atoms per cent O^{18} for a sample containing no atoms per cent excess O^{18} is 0.21 per cent. All three samples were found to contain 0.32 per cent. The theoretical uptake for corticosterone synthesized from DOC in a system containing 1.4 atoms per cent excess O_2^{18} available would be 0.36 atoms per cent or an atoms per cent excess of 0.36-0.21 = 0.15. Table V is a report from Consolidated Electrodynamics Corporation of the mass spectrometry of the CO_2 derived from the pyrolyzed biosynthetic corticosterone.

TABLE V

MASS SPECTROMETER ANALYSES FOR O_2^{18}

Sample Number	Ratio 46/44	Atoms % O^{18}	Atom % Excess	% Theoretical
Sample No. 1 22.1 mg Corticosterone	.00636	.32		
CO_2 Standard	.00410	.21	.11	73
Sample No. 2 22.9 mg Corticosterone	.00638	.32		
CO_2 Standard	.00410	.21	.11	73
Sample No. 3 22.0 mg Corticosterone	.00641	.32		
CO_2 Standard	.00411	.21	.11	73

CHAPTER IV

DISCUSSION

We have shown in this thesis that molecular oxygen is incorporated into the steroid nucleus when corticosterone is enzymatically formed from desoxycorticosterone. Studies on the mechanism of 11_{β}-hydroxylation that were done simultaneously with the work presented in this thesis[54,57] confirm the results that the source of oxygen for 11_{β}-hydroxylation is molecular oxygen. Hayano et al[54] devised an experiment in which desoxycorticosterone was incubated with adrenal homogenate residue in an atmosphere of oxygen enriched with O_2^{18}. They found that 1.05 atom per cent excess O^{18} (theoretical 1.2 atom per cent) was incorporated into the biosynthesized corticosterone. These results are in full agreement with those presented in this thesis that molecular oxygen is utilized directly in the hydroxyl group at the 11_{β} position of corticosterone.

Therefore, it may be taken that enzymic incorporation of molecular oxygen at the 11_{β} position of corticosterone is established. There remains to be discussed the mechanism of this enzymic reaction, particularly the nature of the cofactor requirement and its meaning in terms of the requirement for molecular oxygen.

A. Cofactor Requirements for 11_{β}-Hydroxylation in Various Tissue Preparations

Various tissue preparations have been used in the study of 11_{β}-hydroxylation. It is evident from previous discussion that the cofactors and conditions necessary for 11_{β}-hydroxylation vary and are dependent on the state of integration of the cellular components of the tissue. The tissue preparations that will be discussed are: the intact mitochondria, disrupted mitochondria, acetone powders and the extracts of acetone powders.

Using intact mitochondria Brownie and Grant[34] found that the highest level of 11_{β}-hydroxylation could be obtained in the presence of several of the citric acid cycle intermediates plus ATP or ADP and magnesium ions. They concluded concurrent oxidative phosphorylation was necessary.

Sweat and Lipscomb,[35] incubating mitochondria in 0.04 M sodium phosphate buffer (PH 7.4), 0.004M $MgCl_2$ and 40 micrograms of desoxycorticosterone, noted that fumarate and malate induced higher rates of activity than TPNH or isocitrate and that the glucose-6-phosphate TPN system induced the highest rate of activity of all the cofactors used. It is of interest concerning this last point that Kelly[48] has reported that glucose-6-phosphate dehydrogenase activity is higher in the adrenal cortex than any

other tissue tested. Recently Haynes and Berthet[49] have reported that ACTH added to media containing adrenal slices causes a rapid and specific activation of phosphorylase in adrenal tissue. Increased phosphorylase activity leads to the conversion of glycogen to glucose-6-phosphate. This compound is metabolized primarily by dehydrogenation. In the process of dehydrogenation, TPNH is generated, and this can be utilized to stimulate steroid synthesis by acting as a cofactor in the many hydroxylating reactions in the biosynthetic sequence of adrenal steroids. In my opinion, this most significant system is lost when the integrity of the adrenal tissue is destroyed by homogenization and fractionation.

Hayano and Dorfman[33,38] have used adrenal homogenates prepared in the Waring blendor, an instrument known to disrupt the mitochondria. This preparation was centrifuged at 5000 XG to yield a sediment which was twice resuspended in saline and centrifuged to remove soluble material. The washing process served to diminish the level of endogenous 11_{β}-hydroxylation to such a point that no transformation of desoxycorticosterone was observed unless fumarate and magnesium ions were added.[33] These residue preparations required no added ATP or DPN when fresh. Aged preparations did require both these cofactors or TPN alone.[38] When TPN was used the requirement for

magnesium ions was no longer present indicating the transformation of ATP and DPN to the more specifically required TPN in the presence of this ion.

The fact that 11_{β}-hydroxylation activity is found in acetone powders(38) of the above residue, which known to be deficient in cytochrome oxidase activity, would indicate that this transfer of electrons from TPNH is independent of the cytochrome system. This would also indicate that concurrent oxidative phosphorylation is not an essential factor in the 11_{β}-hydroxylation reaction.

Brownie and Grant,(36) using a KCl extract of dried acetone powders of adrenal mitochondria, found that oxygen was essential to the reaction, that fumarate, malate or isocitrate could be used as generators of reduced TPN, and that TPNH is a definite cofactor.

B. Proposed Mechanisms of 11_{β}-Hydroxylation

Numerous theories have been proposed for the mechanism of 11_{β}-hydroxylation of adrenal steroids. Some of these theories have been tested experimentally and others have been merely postulated on theoretical basis.

On the basis of steric considerations, the 11_{β}-hydroxylation is an interesting reaction since the hydrogen at 11α position is reactive while the 11_{β} hydrogen is chemically unreactive(10) and sterically hindered. One mechanism that has been proposed on the basis of the

increased reactivity of the 11α hydrogen as compared to the 11$_\beta$ hydrogen is as follows: initially there may be an attack on the 11α H of a 11-desoxysteroid to form the 11α OH. This sequence was tested and it was found that the reduction of the 11 ketone to 11$_\beta$ hydroxy does not occur with cortisone as substrate in either adrenal perfusion[13] or adrenal homogenates.[12]

Another speculation on the mechanism of the introduction of a hydroxyl group into the 11$_\beta$ position of a steroid is patterned on an analogous situation, the stepwise transformation of succinate to fumarate to malate involving the obligatory unsaturated derivatives. The possible intermediacy of the $\Delta^{9(11)}$ structure was eliminated by the use of the unsaturated analogs of desoxycorticosterone and 11 desoxycortisol.[11] While $\Delta^{11(12)}$ dehydro DOC has not been tested Dorfman et al[11] have reported that $\Delta^{11(12)}$ dehydroprogesterone is not converted to corticosterone by crude adrenal homogenates which contain both the 11$_\beta$ and C-21-hydroxylation systems. It would thus seem possible that the mechanism of primary dehydrogenation coupled with secondary hydration may not be involved in the 11$_\beta$-hydroxylation reaction.

Fieser[52] has suggested that the biological oxidation at C11 may take the path of formation of a seco derivative of desoxycorticosterone, allylic oxidation at C11, and

ring closure. No evidence for or against this view is as yet available.

Levy et al[16] have suggested a mechanism whereby OH free radicals are involved in the direct oxidation of desoxycorticosterone. In this process the α hydrogen at C11 is removed and a steroid radical is formed which then couples with the hydroxyl radical. Mason[53] made the following comments on this mechanism:

> If free radicals were involved in this system non specific reactions characteristic of radicals would occur but 11_B hydroxylation is both position and stero-specific. For this reason it is likely that consumed molecular oxygen remains under enzymic control until the product is formed.

Additional evidence against the free radical mechanism can be found in the work of Hayano[54] and deBruin[47] where H_2O^{18} enriched water was used without an incorporation of O^{18} into the hydroxylated product.

Previous to these studies utilizing H_2O^{18} Hayano and Dorfman[38] examined the possibility of the addition of water to a precursor of the 11_B hydroxylated steroid by using deuterium oxide. No deuterium in excess of normal abundance was found incorporated in the end product. The experiments by Hayano[54] and deBruin[47] mentioned above confirmed this finding and completely ruled out the participation of water as a source of oxygen in the 11_B-hydroxylation reaction.

The possibility of a peroxidase enzyme system requiring oxygen and TPNH as suggested by Brodie(55) must be considered as a C11-hydroxylating mechanism. This system was studied by deBruin(47) using horseradish peroxidase as the enzyme and desoxycorticosterone as the substrate. This system failed to convert desoxycorticosterone to corticosterone.

With these preliminary results in mind it would appear that the 11_{β} hydroxylating enzyme of adrenal mitochondria would fit in the category of a mixed function oxidase according to the classification of Mason.(53,56)

To identify an enzyme as a mixed function oxidase it is necessary:

1. to determine the amount of oxygen consumed per molecule of substrate transformed,
2. to determine the amount of oxygen incorporated into the product,
3. to determine the source of the incorporated oxygen and
4. to show that the two reducing equivalents are also consumed during the reaction.

It is evident from the preceding discussion that further characterization of the enzyme and determination of the stoichiometric relationship between oxygen, TPNH and substrate are now required to confirm this classification.

Summary

Desoxycorticosterone was incubated in the presence of adrenal mitochondria, fumarate, magnesium, TPN and in an atmosphere of oxygen enriched with O_2^{18}. The corticosterone enzymatically synthesized was subjected to mass spectrometer analysis. An average of 75 per cent theoretical incorporation of O_2^{18} was found. It was concluded from these data that molecular oxygen is utilized directly in the hydroxylation of steroids at the C-11_β-position.

BIBLIOGRAPHY

1. Kendall, E. C., Hormones of the Adrenal Cortex. Endocrinology, 30:853-860, 1942.

2. Wintersteiner, O., Schwenk, E., Hirschmann, H., and Whitman, B., Chemical Nature of Follicular Hormone. J. Amer. Chem. Soc., 58:2652-2653, 1936.

3. Reichstein, T., Shoppee, C. W., The Hormones of the Adrenal Cortex. Vitamins and Hormones, 1:345-413, 1943.

4. Vogt, M., The Output of Cortical Hormone by the Mammalian Suprarenal. J. Physiology, 102:341-356, 1943.

5. Bush, I. E., Species Differences in Adrenocortical Secretion. J. Endocrinology, 9:95-100, 1953.

6. Simpson, S. A., Tait, J. F., Secretion of a Salt Retaining Hormone by the Mammalian Adrenal Cortex. Lancet, 263:226-227, 1952.

7. Hechter, O., Zaffaroni, A., Jacobsen, K. P., Levy, H., Jeanloz, R. W., Schenker, V., and Pincus, G., The Nature and the Biogenesis of the Adrenal Secretory Product. Recent Progress in Hormone Research, 6:215-241, 1951.

8. Stone, D. and Hechter, O., Studies on ACTH Action in Perfused Bovine Adrenals: The Site of Action of ACTH in Corticosteroidogenesis. Arch. Biochem. and Biophys., 51:457-469, 1954.

9. Hechter, O., Solomon, M. M., Zaffaroni, A., and Pincus, G., Transformation of Cholesterol and Acetate to Adrenal Cortical Hormones. Arch. Biochem. and Biophys., 46:201-213, 1953.

10. Zaffaroni, A. and Rubin, B. A., In Vitro Synthesis of Corticosteroids, Discussion. Ciba Foundation Colloquia on Endocrinology, 7:204, 1953.

11. Dorfman, R. I., Hayano, M., Haynes, R. and Savard, K., The In Vitro Synthesis of Adrenal Cortical Steroids. Ciba Foundation Colloquia on Endocrinology, 7:191-200, 1953.

12. Hechter, O., Biogenesis of Adrenal Cortical Hormones. Ciba Foundation Colloquis on Endocrinology, 7:161-173, 1953.

13. Meyer, A., Chemical Transformations of Steroids by Adrenal Perfusion. V, Blood Incubations and Perfusion with Cortisone. J. Biol. Chem., 204:469-487, 1953.

14. Levy, H., Unpublished results.

15. Hechter, O. and Stone, D., Unpublished results.

16. Levy, H., Jeanloz, R. W., Marshall, C. W., Jacobsen, R. P., Hechter, O., Schenker, V., and Pincus, G., Chemical Transformations of Steroids by Adrenal Perfusion. II 11-Desoxycorticosterone and 17-Hydroxy 11-Desoxycorticosterone, J. Biol. Chem., 203:433-451, 1953.

17. Meyer, A., Unpublished results.

18. Jeanloz, R. W., Levy, H., Jacobsen, R. P., Hechter, O., Schenker, V., and Pincus, G., Chemical Transformations of Steroids by Adrenal Perfusion. III Δ^4 Androstene 3, 17 Dione. J. Biol. Chem., 203:453-461, 1953.

19. Hechter, O. and Pincus, G., Genesis of the Adrenocortical Secretion. Physiol. Rev., 34:459-496, 1954.

20. Plager, J. E. and Samuels, L. T., Enzyme System Involved in the Oxidation of Carbon-21 Steroids. Fed. Proc., 11:383, 1952.

21. Plager, J. E. and Samuels, L. T., Factors Affecting Activity of the Enzymes Oxidizing Carbons 17 and 21 of the Pregnene Nucleus. Fed. Proc., 12:357, 1953.

22. Beyer, K. F. and Samuels, L. T., Distribution of Steroid 3_B ol Dehydrogenase in Cellular Structures of the Adrenal Gland. J. Biol. Chem., 219:69-76, 1956.

23. Samuels, L. T., Helmreich, M. L., Laster, M. B., Reich, H., An Enzyme in Endocrine Tissues Which Oxidizes Δ^5-3 Hydroxy Steroids to α-$_B$ Unsaturated Ketones. Science, 113:490-491, 1951.

24. Samuels, L. T., Studies of the Enzymes Involved in the Synthesis and Degradation of the Hormones of the Adrenal Cortex. Ciba Foundation Colloquia on Endocrinology, 7:176-190, 1953.

25. Saba, N., Hechter, O. and Stone, D., The Conversion of Cholesterol to Pregnenolone in Bovine Adrenal Homogenates. J. Amer. Chem. Soc., 76:3862-3864, 1954.

26. Plager, J. E. and Samuels, L. T., The Conversion of Progesterone to 17-Hydroxy-11-Desoxycorticosterone by Fractionated Beef Adrenal Homogenates. J. Biol. Chem., 211:21-29, 1954.

27. Plager, J. E., Samuels, L. T., Synthesis of C^{14}-17-Hydroxy 11-Desoxycorticosterone and 17-Hydroxycorticosterone by Fractionated Extracts of Adrenal Homogenates. Arch. of Biochem. and Biophys., 42:477-478, 1953.

28. Hayano, M., Dorfman, R. I., Prins, D. A., Metabolism of the Steroid Hormones. Conversion of Desoxycorticosterone to Glycogenic Material in Vitro. Proc. of the Soc. for Exp. Biol. and Med., 72:700-701, 1949.

29. McGinty, D. A., Smith, G. N., Wilson, M. L. and Worrel, C. S., The Biosynthesis of 17-Hydroxycorticosterone from 11-Desoxy-17-Hydroxycorticosterone. Science, 112:506, 1950.

30. Savard, K., Green, A. A., and Lewis, L. A., Oxidation of 11-Desoxycorticosteroids with Adrenal Tissue Homogenates. Endocrinology, 47:418-428, 1950.

31. Kahnt, F. W. and Wettstein, A., Die 11-Oxidation von Desoxycorticosteron und Reichstein' Substanz Smit Rilfe Tierischer Organ Homogenate. Bildung von Corticosteron und 17-Oxycorticosteron. Chim. Acta, 34:1791-1805, 1951.

32. Sweat, M. L., Enzymatic Synthesis of 17-Hydroxycorticosterone. J. Amer. Chem. Soc., 73:4056, 1951.

33. Hayano, M. and Dorfman, R. I., The Enzymatic C-11-β Hydroxylation of Steroids. J. Biol. Chem, 201: 175-188, 1953.

34. Brownie, A. C. and Grant, J. K., The in Vitro Enzymic Hydroxylation of Steroid Hormones. Biochem. J., 57:255-263, 1954.

35. Sweat, M. L. and Lipscomb, M. D., A Transhydrogenase and Reduced Triphosphopyridine Nucloetide Involved in the Oxidation of Desoxycorticosterone to Corticosterone by Adrenal Tissue. J. Amer. Chem. Soc., 77:5185-5186, 1955.

36. Grant, J. K. and Brownie, A. C., The Role of Fumarate and TPN in Steroid Enzymic 11$_\beta$ Hydroxylation. Biochem. et. Biophys. Acta, 18:433-434, 1955.

37. Hayano, M., Dorfman, R. I., and Rosenberg, E., Steroid Hydroxylases of the Adrenal. Fed. Proc., 14:224, 1955.

38. Hayano, M., Dorfman, R. I., On the Mechanism of the C-11-$_\beta$ Hydroxylation of Steroids. J. Biol. Chem., 211:227-235, 1954.

39. Mason, H. S., Fowlks, W. L. and Peterson, E., Oxygen Transfer and Electron Transport by the Phenolase Complex. J. Amer. Chem. Soc., 77:2914, 1955.

40. Sweat, M. L., Silica Gel Microcolumn for Chromatographic Resolution of Cortical Steroids. Anal. Chem., 26:1964-1967, 1954.

41. Zaffaroni, A., Absorption Spectra of Sulfuric Acid Chromogen Obtained from Adrenal Steroids and Related Compounds. J. Am. Chem. Soc., 72:3829, 1950.

42. Sweat, M. L., Sulfuric Acid Induced Fluorescence of Corticosteroids. Anal. Chem., 26:773-776, 1954.

43. Wintersteiner, O. and Pfiffner, J. J., Chemical Studies on the Adrenal Cortex III. J. Biol. Chem., 116:291-305, 1936.

44. Zaffaroni, A., Micromethods for the Analysis of Adrenocortical Steroids. Recent Progress in Hormone Research, 8:51, 1953.

45. Doering, W. E. and Dorfman, E., Mechanism of the Peracid-Ketone Ester Conversion. Analysis of Organic Compounds for Oxygen-18. J. Amer. Chem. Soc., 75:5595-5598, c.f., also Unterzaucher, J. Ber., 73 B:391-404, 1940.

46. Dobriner, K., Katzenellenbogen, E. R., Jones, N. R., Infrared Absorption Spectra of Steroids. Interscience Publishers, 1953.

47. de Bruin, C. H., The Role of Water in the In Vitro Enzymic Hydroxylation of 11-Desoxycorticosterone. Thesis 1957, University of Oregon Medical School.

48. Kelley, T. L., Nielson, E. E., Johnson, R. B., and Vestling, C. S., Glucose-6-Phosphate Dehydrogenase of Adrenal Tissue. J. Biol. Chem., 212:545-554, 1955.

49. Haynes, R. C. and Berthet, L., Studies on the Mechanism of Action of the Adrenocorticotropic Hormone. J. Biol. Chem., 225:115-124, 1957.

50. Fieser, L. F. and Fieser, M., Natural Products Related to Phenanthrene. New York: Reinhold, 3rd Ed., 1949. p. 477.

51. Hayano and Dorfman, Unpublished observations.

52. Fieser, L. F., General Discussion. Ciba Foundation Colloquia on Endocrinology, 7:289, 1953.

53. Mason, H. S., Mechanisms of Oxygen Metabolism. Advances in Enzymology, 79:233, 1957.

54. Hayano, M., Lindberg, M. C., Dorfman, R. I., Hancock, J. E. H. and Doering, W. von E., On the Mechanisms of the C-11-β Hydroxylation of Steroids; A Study With H_2O^{18} and O_2^{18}. Arch. Biochem. and Biophys., 59:529-532, 1955.

55. Brodie, B. B., Axelrad, J., Cooper, J. R., Gauderre, L., LaDu, V. N., Choco, M., and Udenfriend, S., Detoxication of Drugs and Other Foreign Compounds by Liver Microsomes. Science, 121:603-604, 1955.

56. Mason, H. S., Mechanism of Oxygen Metabolism. Science, 125:1185-1188, 1957.

57. Sweat, M. L., Aldrich, R. A., de Bruin, C. H., Fowlks, W. L., Heiselt, L. R., and Mason, H. S., Incorporation of Molecular Oxygen into 11β Position of Corticosteroids. Fed. Proc., 15:1200, 1956.

Typed by

Freida M. Smith