THE ROLE OF WATER IN THE IN VITRO ENZYMIC HYDROXYLATION OF 11-DESOXYCORTICOSTERONE

by

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CHAPTER I

INTRODUCTION

Interest in the adrenocortical hormones dates back to 1855 when Addison described the profound alterations of numerous vital body functions associated with cases of adrenocortical insufficiency. The multiplicity of functions of these steroid hormones is now well documented.

Recently it has been recognized[1] that the hormones responsible for the far reaching effects exerted by the adrenal cortex may be roughly divided into two classes on the basis of their physiological activities. One group of steroids functions chiefly as a regulator of carbohydrate metabolism, while the other group helps to govern electrolyte and water balance. These functions have been found to overlap somewhat[2], but such a division is still useful in classification. This difference in function stems from a difference in chemical configuration. The carbohydrate regulating steroids all bear an oxygen atom at C-11, while such a substituent is absent from this position in the steroids regulating electrolyte and water balance. Furthermore, oxygenation at the 11 position seems to be mandatory if the steroid is to exert the clinically observed function

of anti-inflammation and efficacy in the repair of the collagen pathologies[3]. Biochemical interest in the C-11 oxygen grouping thus stems from the fact that its presence on or absence from the proper steroidal nucleus has such a marked effect on the end function of the steroid. This fact, coupled with a growing body of evidence that such disease entities as adrenal hyperplasia[4] and some forms of rheumatic fever[5] are perhaps due to a deficiency in function (or perhaps a 'biochemical lesion') in the 11-β hydroxylating enzyme system of the adrenal gland, suggested that a study of this enzyme system would be biochemically lucrative.

Much of the work done in the broad field of adrenal endocrinology has been accomplished on a strictly clinical dose-effect basis. While this type of approach is useful in that studies of isolated clinical entities often bring results as far as immediate symptomatic amelioration is concerned, they do not contribute as much toward an understanding of the underlying biochemical alterations occurring in disease states as do studies of the actual enzyme systems involved. In addition, the time is now approaching when an investigation of the mechanism of action of an enzyme system supersedes in importance the physical descriptions of fractionated enzyme preparations, so popular in the last decade. Furthermore, a study of enzyme function offers a more thorough comprehension of the nature of bodily

reactions.

Evidence from the literature concerning an adrenal enzyme system catalyzing the C-11-β hydroxylation of steroid substrates will be presented here, and also a brief description of enzymatic steroid reactions related to the above reaction; and a sequential biogenetic scheme will be given.

Pertinent steroid formulae will be found in Figure 1[6] (page 4).

A. History

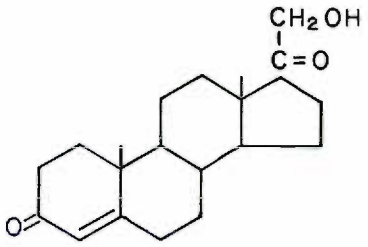
Hechter and Pincus[7], using the technique of adrenal perfusion, showed that added amounts of desoxycorticosterone were converted to a substance with marked glycogenic activity, suggesting that the substance possessed a C-11 oxygen grouping[8]. Soon afterward this substance was shown to be corticosterone[9]. In an experiment making use of whole adrenal homogenate, and using 11-desoxy-17-hydroxycorticosterone as substrate, McGinty[10] was able to effect a conversion to 17-hydroxycorticosterone.

Savard, Green and Lewis[11] obtained evidence for the formation of 11-β-hydroxylated derivatives from both 11-desoxycorticosterone and 17-hydroxy-11-desoxycorticosterone on incubation with adrenal homogenates.

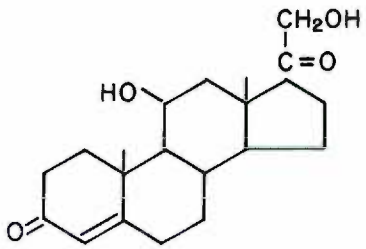
Hayano, Dorfman and Yamada[12] incubated 11-desoxycorticosterone with adrenal homogenate and, in a preliminary

Figure 1

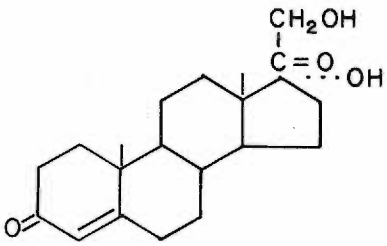
Steroid Formulae Pertinent
to this Thesis



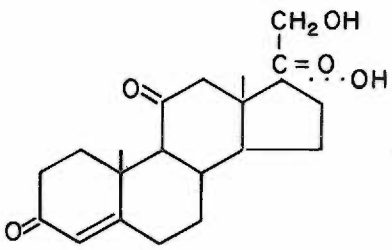
11-Desoxycorticosterone
(DOC)



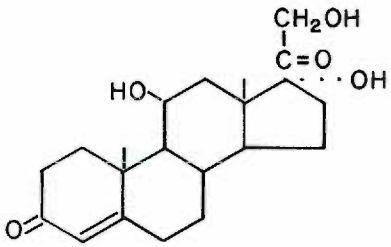
Corticosterone
(Comp. B)



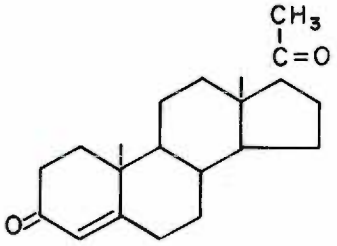
17 α Hydroxy-11-Desoxycorticosterone
(Comp. S)



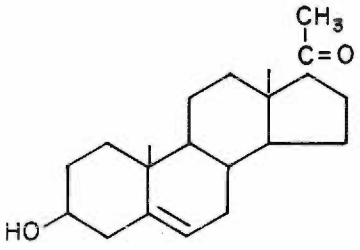
17 α Hydroxy-11-Dehydrocorticosterone
(Comp. E)



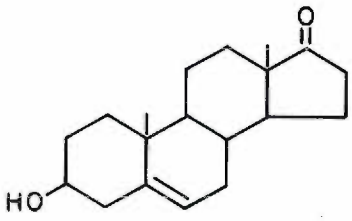
17 α Hydroxycorticosterone



Progesterone



Pregnenolone



Dehydroepiandrosterone

report, identified the product as a substance possessing strong glycogenic action. These workers made the additional observations that ATP*, DPN**, and fumarate enhance the reaction as evidenced by increased yields. They noted also that the reaction is necessarily aerobic and that high levels of 11-desoxycorticosterone substrate inhibit the reaction.

Kahnt and Wettstein[13] confirmed the stimulatory effect of added fumarate in an extended study with dicarboxylic acids, and observed that the optimal stimulatory combination in their whole homogenate preparation was fumarate plus nicotinamide.

Major credit for showing that 11-β-hydroxylation is a function of the mitochondrial fraction goes to Sweat[14], who incubated 17-hydroxy-11-desoxycorticosterone with various adrenal cell fractions and found that most of the activity with respect to the conversion to 17-hydroxycorticosterone could be explained on the basis of the 'insoluble cellular constituents', which were later shown by this author to be composed, in the main, of mitochondria[15]. He also noted that the conversion proceeds best at a pH around the neutral point and at temperatures between 35° and 40° C., with

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*Adenosine triphosphate

**Diphosphopyridine nucleotide

marked decline in activity above 40°.

An analagous conversion involving C-11-β-hydroxylation, in which desoxycorticosterone instead of 17-hydroxycorticosterone was incubated with adrenal mitochondrial preparations, was reported by Hayano and Dorfman[16]. Corticosterone was the substance isolated in this case, and a further requirement for the reaction noted was the presence of magnesium ions. In the same report, these workers showed that certain structural configurations of the substrate have definite effects upon the extent of the observed conversion. Most notably, the presence of a hydroxyl group at carbon 17 has no effect, but the lack of a hydroxyl group at C-21, a reduced A ring, or the presence of a hydroxyl group at C-20 all preclude 11-β hydroxylation.

Brownie and Grant[17] in an intensive study isolated corticosterone from mitochondrial preparations incubated with desoxycorticosterone (DOC) and made a survey of various 'Krebs cycle' acids to determine their effects on this reaction. These authors discovered that (in addition to fumarate) malate, succinate, citrate, oxalacetate and α-ketoglutarate all catalyze the reactions, with α-ketoglutarate exerting the most marked effect. On this basis, these investigators concluded that there is no specific requirement for fumarate, but only for a member of the Krebs citric acid cycle.

Sweat and Lipscomb[18] described additional studies on the specific enzyme system responsible for 11-β hydroxylation and, in a later report[19], showed that further requirements for this conversion of DOC to corticosterone include a reduced TPN* and a transhydrogenase enzyme.

Hayano and Dorfman[20] confirmed the observation of Sweat, i.e., that TPN is a necessary adjunct to C-11-β hydroxylation, when they found that TPN alone could be substituted for DPN and ATP. The obvious conclusion in this case was that TPN was being formed from the added DPN and ATP.

Recent studies by both Grant and Brownie[21] and Hayano, Dorfman and Rosemberg[22] reveal the significant fact that KCl-water-soluble extracts of acetone dried powders from adrenal mitochondrial preparations possess the ability to introduce a C-11-β hydroxyl group into 11-desoxycorticosterone. These water soluble extracts exhibit the same need for fumarate and reduced TPN as did the earlier mitochondrial preparations. The absolute requirement of aerobic conditions was also observed in this case.

While the literature cited above offers evidence for the hydroxylation of the two substrates, desoxycorticosterone

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*Triphosphopyridine nucleotide

and 17-hydroxycorticosterone, numerous investigators have shown the C-11-β hydroxylation of a variety of other steroidal substrates using adrenal gland homogenates. Thus Brownie, Grant and Davidson[23] have shown the 11-β-hydroxylation of progesterone; Hayano and Dorfman[24], the 11-β-hydroxylation of Δ^4-pregnene-6β,17α,21-triol-3,20-dione; and Hayano and Dorfman[25], the 11-β hydroxylation of Δ^4-androstene-3,17-dione. Whether or not these reactions represent physiological realities remains in the field of conjecture, at least in the case of the latter two compounds.

It seems pertinent to mention at this point studies using microorganisms as catalysts of 11, 17, and 21 hydroxylation of various steroid substrates. While these studies admittedly are of somewhat academic interest, they do indicate the presence of similar enzyme systems, and, as such, are worthy of note from a biochemical standpoint. Kahnt et al[26] prepared the C-11-α hydroxy epimers of corticosterone and 17-hydroxycorticosterone, while Peterson and associates[27] developed a technique for the introduction of a C-11-α hydroxyl group in the progesterone molecule using the fungus Rhizopus Mucorales. Shull and Kita[28] biosynthesized corticosterone from 11-desoxycorticosterone through the action of Curvularia Luneata, and in similar experiments McAleer and Dulaney[29] transformed progesterone to 11-desoxycorticosterone using the organism Wojnoicia Graminis, showing a C-21

hydroxylation, while Meister et al[30] were successful in producing the 17-α-hydroxy derivative of progesterone and corticosterone through the use of Cephalothecium Roseum.

B. Related Steroidal Oxidation Systems

Workers interested in adrenal enzyme systems, primarily from a biogenetic standpoint, have obtained evidence for the existence of at least four additional enzyme systems as separate and distinct entities from the C-11-β hydroxylating system. Two of these systems, i.e., the 17-α,21 hydroxylating system and the 3-β-ol dehydrogenase system, provide methods for the synthesis of steroid hormones with a known definite function. The other two systems, the 6-β hydroxylating and the 19 hydroxylating systems, may represent side reactions or, at best, biosynthetic schemes for the production of steroids with an unknown function.

Plager and Samuels[31], using the technique of differential centrifugation, isolated an enzyme system in the soluble supernatant fraction of adrenal homogenates capable of hydroxylating progesterone to 11-desoxycorticosterone. This reaction represents both a 17-α and a 21 hydroxylation. These investigators noted that 21-hydroxylation occurred with added DPN but that the 17-α hydroxylation required both DPN and ATP, suggesting that perhaps 17-α hydroxylation is TPN dependent.

Samuels[32] observed the presence of an enzyme in testis, ovary, placenta, and adrenal cortex, which catalyzes the oxidation of a 3-β-hydroxy group to a Δ^4-unsaturated 3-keto group on a steroid nucleus. Further investigation by Beyer and Samuels[33] indicated that this enzyme system is associated with the microsomal portion of adrenal homogenates fractionated by differential centrifugation. This enzyme system was found to exhibit a requirement for DPN.

Evidence for the presence of an enzyme whose function is hydroxylation at the 6 position of the steroid nucleus is based mainly on studies of urinary metabolites. Information on this enzyme is meager[34], and the significance of the enzyme system is completely unknown.

Hayano and Dorfman[35] reported the conversion of 11-desoxycorticosterone to 19-hydroxy-11-desoxycorticosterone after incubation of the former with adrenal mitochondrial preparations. Levy and Kushensky[36] isolated both 19-hydroxy-11-desoxycorticosterone and 17-α,19-dihydroxy-11-desoxycorticosterone from progesterone perfusate of the adrenal. Interestingly enough, the latter compound was found to have ten times the activity of 11-desoxycorticosterone in the sodium retention test. Other than this, nothing is known of the biological importance of the enzyme system concerned in its formation.

C. Adrenal Steroid Metabolic Reactions

While a complete review of the metabolism or end-degradation of the steroid hormones is obviously beyond the scope of this thesis, it seems well to mention a few generalities concerning adrenal steroid metabolic reactions in the hope of presenting a somewhat more cohesive and clearer picture.

Samuels[38] states that the main site of adrenal steroid metabolism is the liver. This investigator also presents evidence which indicates that kidney and peripheral tissue are not active in this respect. Schneider and Horstmann[37], however, report that reduction of the Δ^4-3 keto grouping of the steroid nucleus, with extensive side chain degradation, occurs when steroids are incubated with liver and with kidney tissue. These workers in a later report[38] noted that the carboxyl group at C-20 was reduced to a hydroxyl group by rat liver enzyme. The production of C_{19} keto steroids from C_{21} steroids was reported by Ganis, Axelrod and Miller[39] in a study involving the incubation of 17-hydroxy corticosterone with kidney tissue. A further observation of this group was that of oxidation of the C-11-β-hydroxyl group to a ketone group. Hubener, Fukushima and Gallagher[40], in an intensive study of the substrate specificity of the C-11,20 keto reduction enzyme systems of rat liver homogenate and cell fractions, found that the microsomal fraction

was the most active and was TPN dependent. They noted also that reduction of an 11 keto group occurred only if a Δ^4 unsaturated 3 keto group was present, indicating that an orderly sequence of events is involved.

In summary, then, it is seen that four main types of reaction are operative in steroid metabolism: (1) reduction of the Δ^4 unsaturated 3 keto grouping to a saturated 3-β-hydroxyl group as recorded by Schneider and Horstmann[37] and by Hechter[41]; (2) reduction of the C-20 group to a hydroxyl group as noted by Schneider[38] and by Hubener et al[40]; (3) degradation of the α ketol side chain of C_{21} steroids to form C_{19} keto steroids as shown by Ganis et al[39] and by Dorfman[42]; and (4) oxidation of active 17-α and 11-β hydroxyl groups to yield C_{17} and C_{11} keto and C_{17} and C_{11} desoxy steroids reported by Ganis et al[39].

In all likelihood, many other metabolic reactions take place in the ultimate degradation of the steroid hormones; however, the four most consistently observed reactions have been reviewed and, in the interest of conciseness, the remaining special cases will be omitted.

D. A General Biogenetic Scheme

For the purpose of clarification and in order better to localize the reference point which the work to be presented in this thesis occupies with respect to the sequential biogenetic scheme, a reaction sequence of corticosteroid

biosynthesis in presented in Figure 2[7] (page 14). In this reaction scheme it is seen that corticosterone and 17-hydroxy corticosterone represent the end products. That such is the case is the considered opinion of Hechter[43], who bases his conclusions on relative amounts of these steroids found in different animal species from adrenal vein blood sampling and on the tenet that doses of ACTH* produce the same relative amounts whether the dosage is minimal or maximal.

Reaction 1, the conversion of acetate to cholesterol, has been observed by many workers, including Srere, Chaikoff and Dauben[44] who followed the conversion of acetate to cholesterol in the adrenal.

Reaction 2, the conversion of acetate to corticosterone and 17-hydroxy corticosterone, was noted by Haines[45] in hog adrenal homogenates. This investigator observed the formation of 17-hydroxy corticosterone, corticosterone, and 17-hydroxy-11-dehydrocorticosterone in the proportion of 92%, 4%, and 4% respectively, and concluded on this basis that 17-hydroxy corticosterone is the predominant hormone in the hog adrenal.

Reaction 3, the direct formation of corticosterone and 17-hydroxy corticosterone from cholesterol, was accomplished by Hechter[43], using bovine adrenals and perfusing them with

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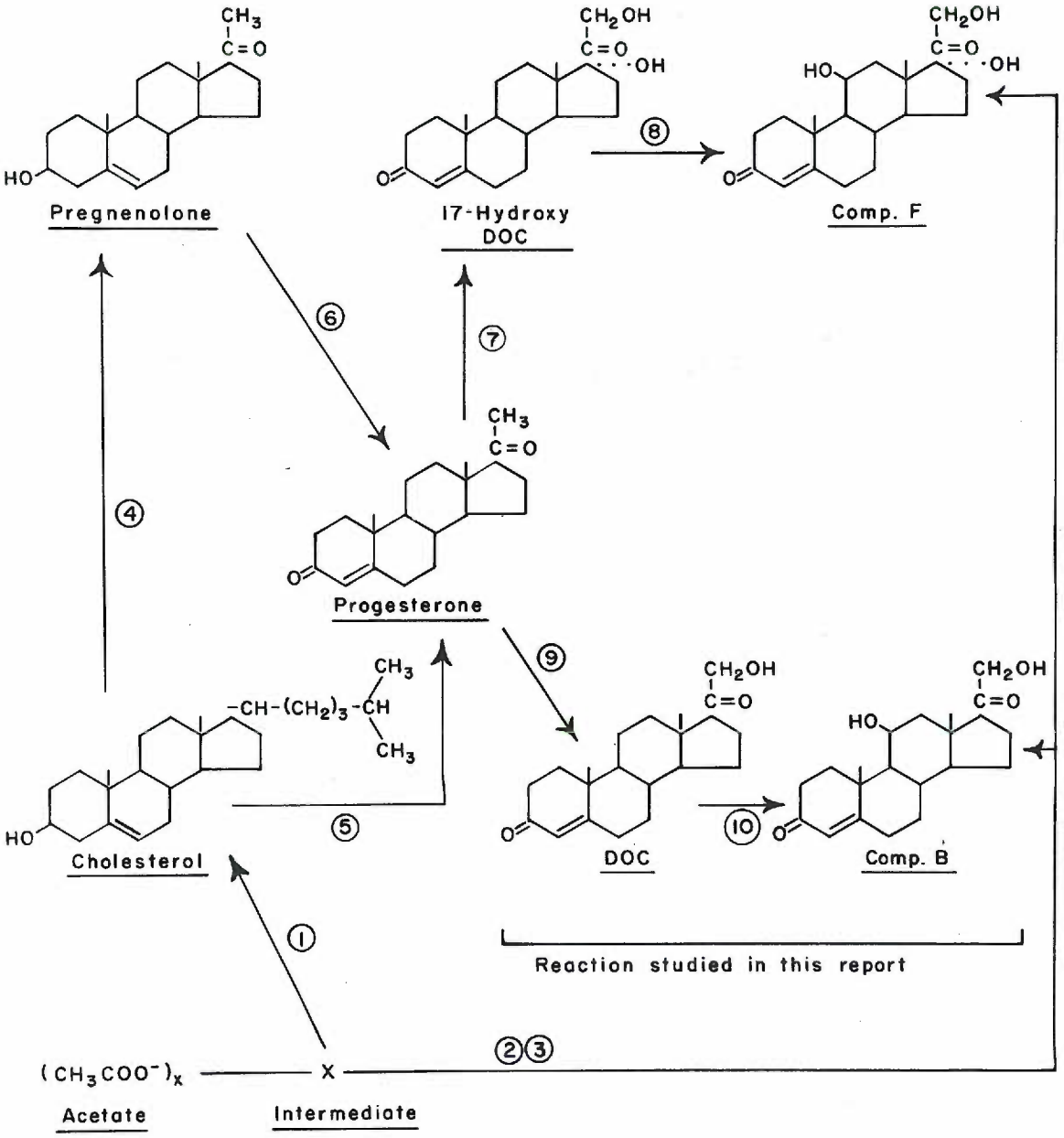
*Adrenocorticotrophic hormone

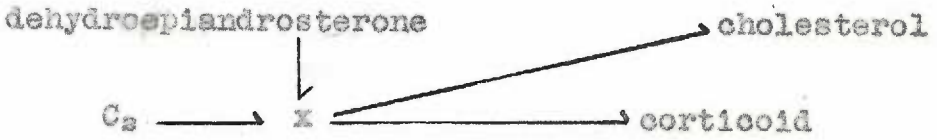
Figure 2

Reaction Sequence of Compound F
and Compound B Synthesis

BIOGENETIC SCHEME



radioactive cholesterol. This worker noted that the specific activity of the corticosterone formed from acetate was higher than that of the cholesterol formed concomitantly and postulated that perhaps a common precursor is involved. This investigator envisaged a scheme whereby acetate condenses to either cholesterol or a steroid precursor in a manner similar to the condensation of C_2 units in the synthesis of squalene. The cholesterol then remains as such or is converted to steroids through a precursor. By way of confirming this condensation, Hechter cites proof for a C_2 unit condensation with dehydroepeandrosterone (DHA), a C_{19} steroid, to form 17-hydroxy corticosterone. Moreover, DHA has been shown to be a normal constituent of human adrenal slices by Bloch, Dorfman and Pincus[46] in a late report. These relationships are summarized in the scheme diagrammed below[7].



Reaction 4, the conversion of cholesterol to pregnenolone, has been shown to occur by Saba, Hechter and Stone[47], using cholesterol C^{14} and bovine adrenal homogenates. These investigators caution, however, against considering adrenal gland cholesterol as an homogeneous 'pool' and cite the importance of both exogenous cholesterol and acetate as precursors.

Reaction 5, the direct formation of progesterone from cholesterol, has been effected by Staple, Lynn and Gurin[48]. Both progesterone and pregnenolene were isolated by this group, using an enzyme preparation found in the supernatant fraction of adrenal gland homogenate. The degraded side chain of cholesterol was identified as isocaproic acid, with trace amounts of propionic acid.

Sequence 6, the conversion of pregnenolone to progesterone, has been accomplished through the use of an enzyme system associated with the microsomal fraction of adrenal homogenates by Beyer and Samuels[33].

Sequence 7, the transformation of progesterone to 17-α-hydroxy-11-desoxycorticosterone, was effected by means of an enzyme system present in the soluble supernatant fraction of adrenal homogenate by Plager and Samuels[31].

Reaction 8, the conversion of 17-hydroxy-11-desoxycorticosterone to 17-hydroxy corticosterone, was originally noted by McGinty et al[10], using adrenal homogenate, and has since been intensively studied by numerous other workers[11,14].

Reaction 9, the formation of 11-desoxycorticosterone from progesterone, was studied by Plager and Samuels[31], using a soluble enzyme system present in the supernatant fraction of adrenal homogenate. These workers indicated in this report that the 17-α hydroxylation seems to be TPN-linked, while the 21 hydroxylation involved in the produc-

tion of 11-desoxycorticosterone proceeds well in the presence of DPN.

Reaction 10, the production of corticosterone from 11-desoxycorticosterone, which forms the basis of this thesis, was initially observed by Hechter and Pincus[7] and has since been exhaustively investigated[5,11,13,16,17,18,19,21,22].

Thus it is seen that each of the steps from acetate to the end products 17-hydroxy corticosterone and corticosterone can be accounted for. Evidence for the stepwise transformation has been adduced from studies involving both adrenal perfusion and incubation of the proper substrate with adrenal homogenates. With respect to the work with adrenal homogenates, some interesting conclusions have been reported: 17-α hydroxylation can occur only if the steroid substrate is not first hydroxylated at C-21[21], and 11-β - hydroxylation will not proceed if the substrate possesses a 21-desoxy group. Conversely, 17-hydroxylation and C-21 hydroxylations do not proceed well if the substrate is first 11-β -hydroxylated[7]. Briefly then, the sequence must be: C-17 hydroxylation ⟶ C-21 hydroxylation ⟶ C-11-β hydroxylation; in addition, ring A oxidation to a Δ^4 unsaturated 3 keto group must precede all steps. For the production of 17-hydroxy corticosterone, then, from the 'key' molecule progesterone, the sequence would be: (1) C-17 α hydroxylation to form 17-hydroxy-progesterone; (2) C-21

hydroxylation to form 17-hydroxy-11-desoxycorticosterone; and (3) and 11-β hydroxylation to produce 17-hydroxycorticosterone. For the synthesis of corticosterone from progesterone the sequence would be: (1) C-21 hydroxylation to form 11-desoxycorticosterone, and (2) 11-β hydroxylation to form corticosterone. The evidence presented above indicates why corticosterone is not converted to 17-hydroxycorticosterone and offers additional proof for the statement of Hechter[43] to the effect that 17-hydroxycorticosterone and corticosterone may be regarded as 'end' products in adrenal gland biosynthetic processes.

Of the three extremely important hydroxylation reactions, i.e., C-21, C-17-α, and C-11-β, the last is by far the most interesting. In the first place, it represents the last stage in the biosynthetic processes culminating in the formation of active steroid hormones. Secondly, it has proved to be the most problematical from the standpoint of co-factor requirements and tissue preparation. Thirdly, the implication of the involvement of this enzyme system in two well recognized disease states[4,5] suggests that an investigation into this system might conceivably bring reward on a clinical plane. Fourthly, the nature and location of the system, functioning as it does within the mitochondria, closely associated with other enzyme systems and with definite requirements for a number of co-factors, advance the

idea that a study of the mechanism of action of this enzyme entity might serve to add to the growing body of knowledge concerning fundamental biochemical processes occurring in the adrenal gland.

Accordingly, a method for determining the source of oxygen utilized by the enzyme system in the process of 11-β hydroxylation was devised, making use of the heavy isotope of oxygen, in the hope that information would be gained as to enzyme type and mechanism of action. In addition, to test the hypothesis that catalase or peroxidase might function as non-specific hydroxylating agents, experiments were undertaken with purified catalase and peroxidase preparations in an attempt to duplicate the 11-β hydroxylation observed, using an artificially constructed system.

These studies form the basis of this thesis.

CHAPTER II

MATERIALS AND METHODS

A. Preparation and Isolation of Mitochondria from Adrenal Glands

Adrenal mitochondria were isolated in the following fashion: bovine adrenal glands were obtained from a local abattoir, usually within 30 minutes after slaughter. The glands were collected on crushed dry ice for freezing in transit and placed in a deep freezer at -40° C. for 24 hours before using. Following this freezing period, the glands were allowed to thaw at 4° C. in a cold room, and the excess fat and connective tissue were stripped off, taking care to avoid laceration of the glands themselves. The glands were then ground to a mince in a household type meat grinder and the mince weighed. 150 to 180 g. was the usual amount utilized. The mince was taken up in 750 ml. of distilled water and homogenized in a Waring blender for seven seconds. The resulting suspension was filtered through two layers of cheesecloth, and the filtrate was centrifuged at 300x G. for five minutes in a refrigerated Sorvall centrifuge to remove larger tissue particles and cellular debris.

The supernatant was removed by gentle suction into a large Erlemeyer flask, decanted into lucite tubes, and recentrifuged for 20 minutes at 17,000-25,000 G. The supernatant was removed as before into a suction flask, care being taken to avoid the settling of fat droplets on the mitochondrial pellet; the supernatant was discarded.

The packed mitochondrial paste remaining in the centrifuge tube was removed with a broad bladed spatula and placed in a Potter-Elvehjem glass homogenizer and resuspended in 72 ml. of H_2O^{18} enriched water*, containing 1.4 atoms percent excess H_2O^{18}.

B. Incubation with H_2O^{18}

Twenty-eight milligrams of desoxycorticosterone (DOC), obtained from Merck and Co., was taken up in one milliliter of absolute ethanol, dried under a stream of nitrogen, and the process repeated. To this solution of DOC and ethanol was added a 60 ml. H_2O^{18} solution of 31.4 mg. NaH_2PO_4, 138.8 mg. Na_2HPO_4, 97.5 mg. $MgCl_2$, 25 mg. triphosphopyridine nucleotide (TPN)**, and 174 mg. of sodium fumarate. The sodium fumarate was prepared from fumaric acid by titration with 1N NaOH to a phenol red end point and

*Stuart Oxygen Co., San Francisco, California.

**Pabst Laboratories, Division of Pabst Brewing Co., Milwaukee, Wisconsin. (100% purity)

subsequent lyophilization of the neutralized solution to a dry powder. To this mixture was added the 72 ml. of adrenal mitochondrial suspension; total volume, 132 ml.

This medium was incubated at 37° C. for three hours with vigorous stirring effected by means of a magnetic stirrer. The reaction was carried out in a 500 ml. Erlenmeyer flask to insure a good surface area/volume ratio, and the gas phase was air. Final pH of the solution was 7.4.

C. Extraction

Following the incubation period, the entire reaction mixture was transferred to a round bottom flask and frozen in a bath of dry ice and methyl cellosolve. The frozen mixture was then lyophilized to a dry powder and the H_2O^{18} obtained from this procedure collected for subsequent distillation. The dry powder was suspended in 130 ml. of distilled water and homogenized in a Waring blender. This water suspension was extracted three times with 150 ml. portions of double-distilled chloroform by homogenizing the water suspension and the chloroform together in a Waring blender fitted with a small blending head. The chloroform-water emulsion was separated by centrifugation, and removal of the chloroform was effected by means of a serum lifter*.

*Zoo-Line Manufacturing Co., Salt Lake City, Utah.

The chloroform extracts were combined, filtered through Reeve-Angel No. 202 open texture filter paper, and taken to dryness *in vacuo* on a water bath at 50° C. The chloroform residue was then partitioned between 20 ml. of 70% ethanol and 100 ml. hexane to remove excess lipid, and the 70% ethanol fraction returned to the chloroform residue flask. The partitioning operation was repeated with a fresh 100 ml. of hexane and the 70% ethanol fraction collected in a 125 ml. Erlemeyer flask. The original chloroform residue flask was rinsed with 5 ml. of 70% ethanol, and the combined ethanol fractions were taken to dryness *in vacuo* on a water bath at 50° C. This semi-dry residue was then lyophilized to complete dryness to avoid any water contamination of the chromatographic column in the subsequent separation and purification.

D. Recovery of the H_2O^{18} Enriched Water

The 'heavy water' recovered from the lyophilization of the reaction mixture was first centrifuged to remove the turbidity and filtered through Reeve-Angel No. 202 open texture filter paper. This filtrate was then distilled in a system designed to avoid dilution of the H_2O^{18} with atmospheric water vapor. The distillation apparatus consisted of a flat bottom flask, a Vigreaux tube, a side arm connected to a West-type condenser, a side arm connected to a calcium chloride tube, and a receiving flask. Heat for the

system was furnished by a small mantle. The recovered water was carefully sealed in glass stoppered bottles, and 3 ml. aliquots were separately sealed for analysis of H_2O^{18} content.

E. Construction of Column

The column which was used in separating and purifying the biosynthesized steroid was constructed from a 50 ml. glass stopcock burette which was cut off at the 32 ml. mark. A water jacket for keeping the column at a constant temperature was constructed from a discarded Van Slyke apparatus water jacket. The column was prepared for use by inserting in order: a glass wool plug, 2 g. of washed reagent grade sand, a fitted piece of Whatman No. 2 filter paper, 3 g. of specially prepared silica gel, another fitted piece of Whatman No. 2 filter paper, and another small glass wool plug.

The silica gel, prior to use in the column, was first washed with water, twice washed with absolute methanol, and dried in a drying oven for 24 hours at 130° C. to remove the methanol. Activation of the silica gel was accomplished by heating in a muffle furnace for three hours at 500° C. This gel was added to the column in the form of a chloroform slurry and permitted to settle by gravitational force. At no time was the column allowed to run dry.

The column was checked for efficiency of function prior to its use in separating the biosynthesized steroid by adding

known amounts of both DOC and corticosterone (Compound B) and comparing the amounts eluted off with the known amounts added. The DOC was eluted off quantitatively in the two 10 ml. 1% ethanol-in-chloroform washes, and the Compound B in the first 15 ml. 5% ethanol-in-chloroform wash. The average yield of DOC in three runs was 96%; average yield of Compound B in three runs was 97%.

The column was also checked for separatory capacity by adding known amounts of DOC and checking for contamination in the 5% ethanol-in-chloroform fractions, and by adding known amounts of Compound B and checking for contamination in the 1% ethanol-in-chloroform fractions. None occurred in either case. These data are found in the following table:

TABLE I

	DOC			Compound B		
Run No.	Added Mg.	Recovered Mg.	%	Added Mg.	Recovered Mg.	%
1	25	24.5	98	25	25.0	100
2	25	24.2	97	25	24.2	97
3	15	14.0	93	15	14.1	94
		Average	96		Average	97

F. Separation and Purification

The residue contained in the 70% ethanol fraction was separated from residual DOC substrate and purified by means of column chromatography, according to the method of Sweat[49]. This method makes use of activated silica gel as the adsorbent and varying concentrations of ethanol in chloroform as the eluent. In this system, the ethanol is the stationary phase, and the chloroform is the mobile phase. The 70% ethanol residue was taken up in 2 ml. of chloroform, placed on the column by means of a micro pipette, and washed onto the silica gel with an additional 2 ml. of chloroform which had been used to rinse the residue flask. The column was then successively washed with two 10 ml. portions of chloroform, two 10 ml. portions of 1% ethanol-in-chloroform (V/V), two 15 ml. portions of 5% ethanol-in-chloroform (V/V), and two 15 ml. portions of 7% ethanol-in-chloroform (V/V). These fractions were collected, and two-drop aliquots of these fractions were collected separately for concentrated sulfuric acid chromogen tests[50]. The fractions collected were taken to dryness under a gentle air stream on a water bath of 50° C., and the tubes containing the residue from the two 5% ethanol-in-chloroform fractions were filled with N_2 gas and sealed for future purification procedures. The two drop aliquots were dried under a direct air stream, taken up in six drops of absolute

ethanol, and to each was added one ml. of concentrated H_2SO_4. These aliquots were then shaken and viewed under an ultra violet lamp, a bright yellow-green fluorescence indicating the presence of corticosterone and serving as a general check on the efficiency of the column. All of the corticosterone synthesized was invariably found in the first of the 15 ml. 5% ethanol-in-chloroform fractions on the basis of the acid chromogen test; consequently, purification procedures were limited to the white crystalline residue remaining in this flask.

Purification consisted of recrystallization from benzene, and re-chromatography on the silica gel column. Recrystallization: 8 drops of benzene were added to the white residue, shaken, heated gently on a water bath at 40° C., 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 crystals were then taken up in 2 ml. of chloroform and re-chromatographed on the silica gel column as previously described. The tube containing the crystalline residue from the first 15 ml. 15% ethanol-in-chloroform wash (the only residue, in this case) was filled with N_2 gas and sealed for use in the identification procedures which follow.

G. Identification

Identification of the biosynthesized material was based on five separate procedures, namely: (1) the sulfuric acid chromogen test of Sweat[50], (2) melting point determinations, (3) ultra violet absorption curves[51], (4) infra red absorption curves, and (5) paper chromatography[52].

1. The addition of conc. H_2SO_4 to corticosterone produced a bright green color in ordinary light, which under ultra violet light fluoresced as a bright yellow-green color. The addition of H_2SO_4 to DOC produced no color, or a slight brown color in visible light, fluorescing only to a faint extent under ultra violet light to give a dull green[53]. This test was used throughout as an index of the progress of the reaction, and also as a general test for the final product since, according to Sweat[54], it is the only steroid which under these conditions exhibits this vivid green fluorescence in ultra violet light.

2. Melting points on the five samples were taken, and mixed melting points with the five samples plus authentic corticosterone (Merck and Co.) were made. These were all made on an Eimer and Amend hot stage type melting point apparatus using an 'A-C' dial setting at 16 volts. The rate of temperature rise was two degrees per minute, and all samples were taken with the biosynthesized material between two glass cover plates.

3. Sulfuric acid chromagen absorption spectra were obtained by the method of Zaffaroni[51]; a three milligram sample of the biosynthesized material was weighed and placed in a clean dry 100 ml. graduated cylinder fitted with a glass stopper; to this was added 100 ml. of concentrated H_2SO_4. Following thorough mixing, the sample was incubated at room temperature for one hour. An authentic 3 ml. sample of corticosterone was weighed and incubated in the same fashion. A 0.5 ml. aliquot of each sample was poured into fused silica micro absorption cells, and optical density readings were made between 220 and 600 mμ in a Beckman Model DU spectrophotometer against a sulfuric acid blank. The slit width was used to adjust the optical density readings to zero with respect to the sulfuric acid blank, thus keeping the sensitivity of the instrument in the middle range. The readings were plotted on standard graph paper: wave length against optical density. Final concentration of steroid material was 15 μg. per 0.5 ml.

4. Infra red absorption spectrometry was performed by the Consolidated Electrodynamics Corporation of Pasadena, California, using a Perkin-Elmer Model 21 double beam infra red spectrometer with a coarse pen tracer. Tracings of an authentic sample of both corticosterone and DOC were made for comparative purposes. The tracings were made covering the range from 650 to 5,000 cm^{-1}.

5. The biosynthesized product was compared with a known sample of corticosterone by means of paper chromatography using the technique of Zaffaroni[52]. Both the ascending and descending techniques were utilized. Two inch strips of Whatman No. 3 filter paper were washed in distilled de-ionized water to remove fluoragens in the paper and ions which might occlude steroid migration; the strips were then hung on stainless steel clips and air dried. The dried strips were immersed in a one-to-one mixture of propylene glycol and ethanol, blotted with large sheets of blotting paper, then air dried until all the ethanol had evaporated. Samples of the biosynthesized material and commercial corticosterone standard were applied to a ruled starting line with micro pipettes, and a hot air drier was used to keep the spots delineated to a diameter of 0.5 cm., or less. The strips were hung in a sealed glass tank containing toluene. A large sheet of No. 3 Whatman filter paper, in contact with the toluene, provided a saturated atmosphere. The strips were left in the sealed tank for one hour to equilibrate. At the end of this period the strips in the ascending system were placed in contact with the toluene at the bottom of the tank, with the starting line at the bottom; the strips in the descending system were placed in contact with the toluene at the top of the tank, with the starting line near the trough. The strips were thus developed for three and one-half hours, removed, and

air dried. Two strips, each containing the reference standard and the biosynthesized corticosterone, were examined under ultra violet light. Two corresponding strips were immersed in a solution containing one part 0.2% aqueous solution of triphenyl tetrazolium chloride and two parts 10% sodium hydroxide solution, rinsed in water, dried, and examined under visible light.

The spots on the untreated strips were visible under ultra violet light as darker than the paper, due to absorbence; the spots on the treated strips were visible in ordinary light as red areas, due to the formation of colored formazan derivatives of the steroids present.

H. Unterzaucher Pyrolysis

In order to ascertain whether or not there was any uptake of O^{18} from the solvent H_2O^{18} of the incubation mixture by the biosynthesized compound, three pooled samples were pyrolyzed in an Unterzaucher pyrolysis apparatus[55] to CO_2, which was trapped in a thin evacuated glass tube and sealed. Mass spectrometry was performed on these samples by the Consolidated Electrodynamics Corporation to determine the atoms per cent excess of O_2^{18} present as CO_2^{18}.

I. Experiments with Catalase

Using the method of La Du and Zannoni[56], 0.5 ml. of crystalline beef liver catalase (obtained from Dr. Nishita,

Department of Biochemistry, University of Oregon Medical School) was incubated in a wide mouth 500 ml. Erlemeyer flask with 2 ml. of 0.2M phosphate buffer (pH 6.4), 40 μM of glutathione neutralized to pH 7.0 by the drop-wise addition of 0.1N sodium hydroxide, 200 γ of 2-6 dichlorophenolindophenol, and 400 μM of DOC dissolved in one ml. of propylene glycol. The incubation time was one hour; the temperature of the reaction was 37° C. Vigorous stirring was effected by means of a magnetic stirrer, and the gas phase was air. The entire incubation mixture was extracted with chloroform as previously described (page 22), dried, partitioned between hexane and 70% ethanol, redried, lyophilized, and subjected to column chromatography. All elutions from the silica gel column were checked for the presence of corticosterone by the sulfuric acid chromogen test of Sweat[50].

Modifications in this basic method in subsequent experiments included the substitution of: (1) 40 μM of dihydroxy fumaric acid for the 40 μM of neutralized glutathione; (2) 40 μM of sodium fumarate for the 40 μM of dihydroxy fumaric acid; (3) 40 μM of neutralized dihydroxy fumaric acid for the unneutralized dihydroxy fumaric acid used previously; (4) one ml. of absolute ethanol for the one ml. of propylene glycol as a solvent for the DOC substrate. A fifth modification was the addition of rat liver

homogenate suspended in 0.88 M sucrose solution to the original system.

J. Experiments with Peroxidase

To 33 mg. of DOC dissolved in one ml. of absolute ethanol were added 25 ml. 0.1 M acetate buffer (pH 5.0) and 60 mg. of one-half neutralized dihydroxy fumaric acid, to a total volume of 30 ml. Oxygen was bubbled into the mixture, and vigorous stirring was accomplished through the use of a magnetic stirrer. Ten micrograms (0.5 ml.) of horseradish peroxidase (purified by D. Keilin) were quickly blown into the mixture, which was then incubated for 20 minutes at 25° C. The entire reaction mixture was then extracted with chloroform, taken to dryness, partitioned between hexane and 70% ethanol, dried, chromatographed on a silica gel column, and checked for corticosterone content by using the acid chromogen test.

Variations in this basic procedure included: (1) the one-half neutralized dihydroxy fumaric acid was changed to completely neutralized dihydroxy fumaric acid; (2) the pH of the reaction was altered from the original pH 5 to pH 4, and then to pH 6; (3) propylene glycol was used to dissolve the DOC in place of ethanol; (4) six drops of Tween 85 were added to the solution of DOC prior to the addition of the aqueous buffer solution in an attempt to produce a colloidal

suspension of the DOC and to prevent possible precipitation of this substrate; (5) the incubation time was increased from 20 minutes to one hour; (6) the quantity of enzyme used was doubled (from 10 to 20 μg.).

On the basis of what appeared to be a positive acid chromogen test for corticosterone in a peroxidase experiment incorporating modifications (1) through (4) above, the following procedure was performed as a confirmatory measure. The chloroform extraction residue was partitioned between hexane and 70% ethanol and the ethanol taken to dryness; the ethanol residue was taken up in 2 ml. of absolute ethanol and 100 γ of this solution were placed on duplicate two inch wide strips of No. 3 Whatman filter paper and chromatographed against an authentic sample of both corticosterone and DOC in an ascending system. Following a three and one-half hour developing period, the strips were removed from the chromatography tanks, dried, and examined under ultra violet light to locate the areas of migration of the unknown residue. The spots containing this unknown compound were cut out, cut into small pieces, and soaked in 50 ml. of absolute methanol for two hours. The methanol was filtered and taken to dryness in vacuo on a water bath of 50° C. Ten ml. of conc. H_2SO_4 were added to this residue, thoroughly mixed, and incubated for one hour. Optical density readings were made on 3 ml. of this solution against a sulfuric

acid blank at wave lengths from 220 to 600 mμ in a Beckman Model DU spectrophotometer and the results graphed, optical density against wave length.

CHAPTER III

RESULTS

A. The Conversion of Desoxycorticosterone to Corticosterone by Adrenal Homogenates

(1) Criteria for Determining the Conversion

Two preliminary tests were used to determine whether or not any conversion from DOC to corticosterone by the adrenal mitochondrial preparation had taken place. The first was the appearance of a white crystalline residue upon evaporation of the solvent in the first 15 ml. 5% ethanol-in-chloroform fraction following extraction, partitioning, and column chromatography of the incubation mixture. The second was the development of a bright yellow-green fluorescence which occurred whenever corticosterone was present upon the addition of concentrated H_2SO_4 and visualization under ultra violet light.

(2) Effect of pH

A series of five incubations was made with completely negative results as regards conversion from DOC to Compound B, at which time it was discovered that the final incubation pH was approximately 4. Accordingly, another series of five incubations was made, in which the pH was

gradually raised over a range from 4 to 8 by altering the phosphate buffer constituent. The conversion began to increase at pH 5, as evidenced through use of the aforementioned qualitative tests, to a maximum yield at 7.4. These data are reported in Figure 3 (page 38) and agree well with the information provided by Hayano and Dorfman[18].

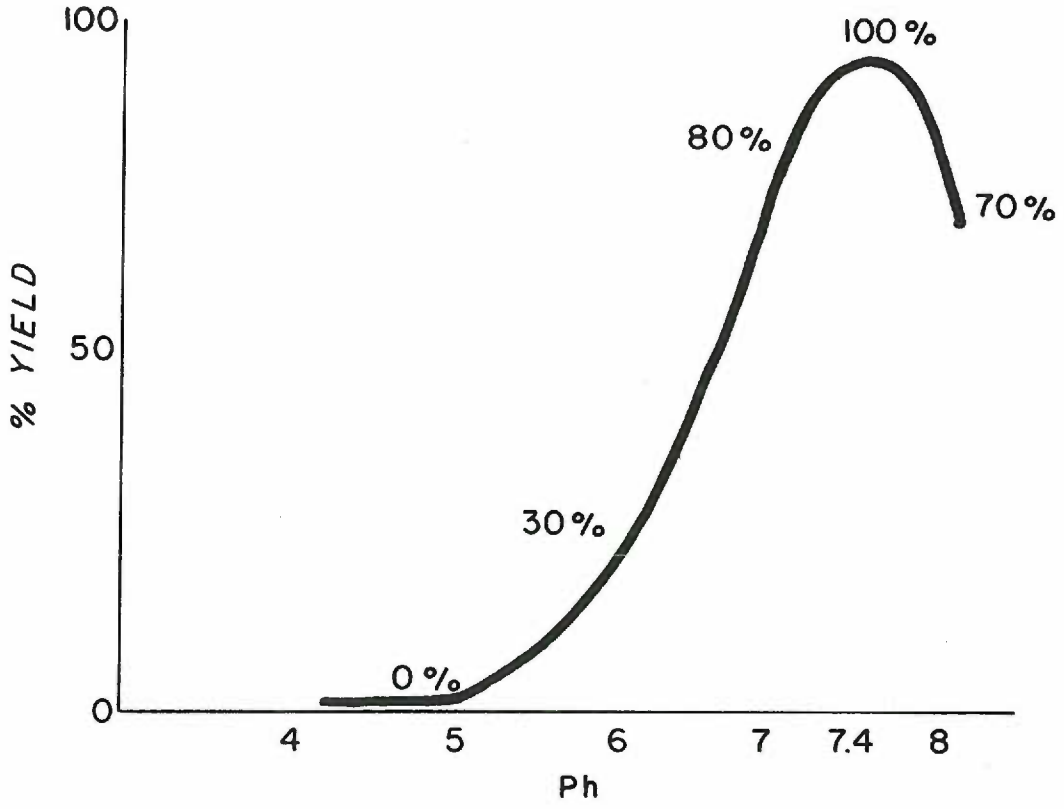
(3) Effect of Dilution

Figure 4 (page 39) graphs an attempt to increase further the percentage conversion by running a series of incubations while varying the dilution of the incubation mixture. Conversion was maximal at total volume (i.e., mitochondria plus buffer) diluted with three volumes of buffer. However, since these studies were being made with heavy water, it was considered best to overlook this advantage in view of the fact that the water was only 1.4 atoms excess per cent to begin with, and further dilution might interfere with mass spectrography.

From the data furnished by five incubations, the average conversion of corticosterone from DOC was 76.5%. The general tendency of the per cent conversion to increase is reflected in Table II (page 40) as the concentration of of substrate decreases. This substrate inhibition was nicely illustrated in a study by Sweat[57].

Figure 3

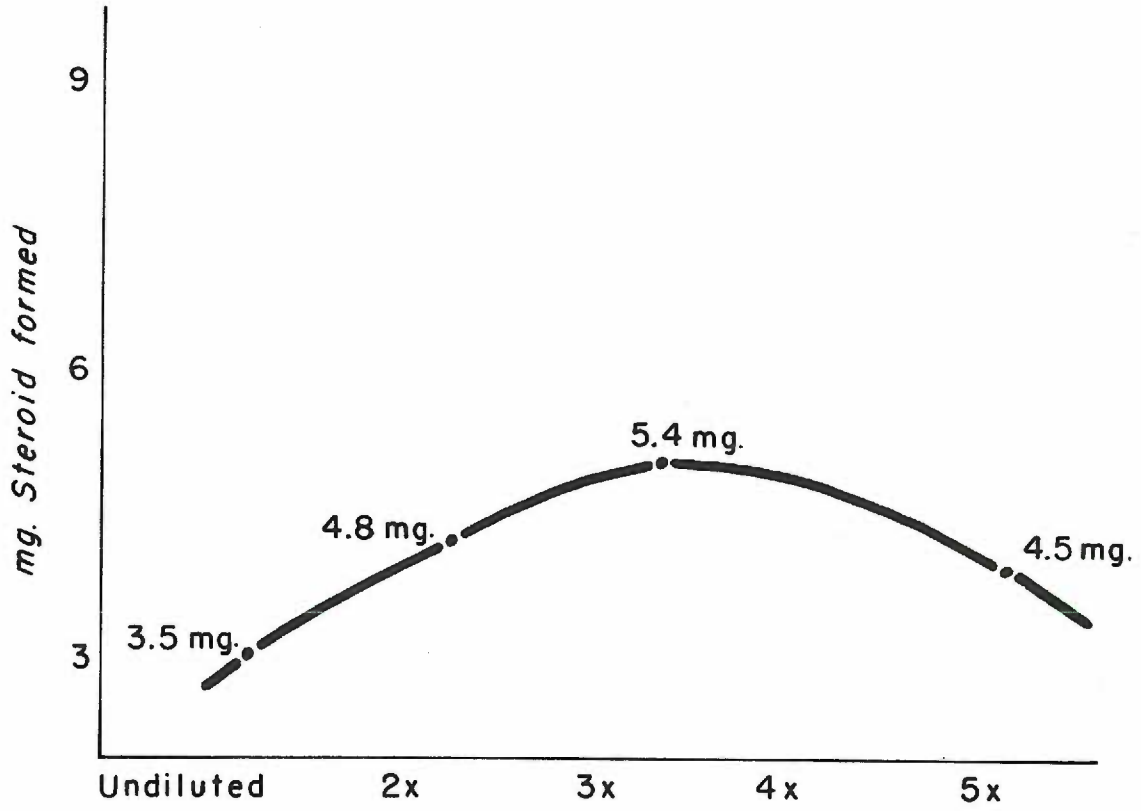
EFFECT OF Ph ON CONVERSION OF 'DOC' TO CORTICOSTERONE



Based on a maximum yield of 79% = 100% of theory

Figure 4

EFFECT OF DILUTION ON % CONVERSION OF 'DOC' TO CORTICOSTERONE



Final volume (i.e. mitochondria + buffer & cofactors)
Diluted with x times buffer

TABLE II

Conversion of Desoxycorticosterone to Corticosterone

Incubation Number	Substrate DOC (mg.)	Compound B Recovered (mg.)	% Conversion
1	28	21.2	75.7
2	28	20.8	74.2
3	25	19.0	76.0
4	25	19.4	77.6
5	25	19.8	79.2
		Average	76.5

B. Characterization of the Biosynthesized Steroid

(1) Sulfuric acid chromogen tests were carried out on each of the five reported samples. The procedure consisted, as previously described, of taking a small sample of steroid up in six drops of absolute methanol and adding to it one ml. concentrated H_2SO_4. The production of an intense yellow-green fluorescence under ultra violet light was taken as indicative of the presence of corticosterone. The results in the case of these five samples were all uniformly affirmative for the presence of corticosterone.

(2) Melting points were taken on each of the five samples and on a pooled mixture of all five samples, on an Eimer-Amend hot stage type of apparatus. In addition, a

mixed melting point was taken on a sample composed of the reference standard plus a mixture of the five samples. That the samples were of the same composition as the reference standard is shown by the fact that the melting points are the same and not depressed by admixture. These data are presented in Table III below. All values are uncorrected. The melting point of corticosterone is given[58] as 180-181° C.

TABLE III

Melting Points of Biosynthesized Steroid Samples

Sample	M.P. (1)	M.P. (2)	Average
Run 1	178°	180°	179°
Run 2	180°	178°	179°
Run 3	177°	179°	178°
Run 4	178°	180°	179°
Run 5	179°	180°	180°
Runs 1 through 5 mixed	178°	179°	179°
Authentic Corticosterone	180°	180°	180°
Authentic Corticosterone * Runs 1-5 pooled	179°	179°	179°

(3) Ultra violet absorption spectra were made on all samples, using a reference standard sample with each for comparative purposes. The samples were read against a concentrated sulfuric acid blank. The absorption maxima of

the biosynthesized steroid were identical in shape and position with those of the standard, occurring at 285, 330, 375, and 455 mμ, with troughs at 230, 355, and 410 mμ. The only difference in the two curves was a vertical displacement due to a small discrepancy in relative concentrations. Figure 5 (page 43) summarizes the information and is presented as being typical of the determinations done on each sample. Figure 6 (page 44) shows the same results obtained on a mixed sample of all five samples read with the standard against the acid blank. Again, the only difference between the curve exhibited by the biosynthesized steroid and the authentic steroid is that of a vertical concentration displacement.

(4) Infra red absorption spectra were done on a pooled sample representing all five incubations, on an authentic sample of Merck corticosterone, and on a sample of the desoxycorticosterone. These spectra were made by the Consolidated Electrodynamics Corporation on a Perkin-Elmer Model 21 double beam infra red spectrometer. From the data presented in Figures 7 and 8 (pages 45, 46) it can be seen that the absorption bands of the synthesized and authentic samples are identical. Figure 9 (page 47) is an infra red absorption spectrum of DOC. The region of importance with respect to corticosterone lies in the middle of the absorption band of atmospheric water. However, a

Figure 5

Absorption Curve of a Single Sample of Biosynthesized Corticosterone and an Authentic Sample of Corticosterone, in Concentrated Sulfuric Acid

Note: Ordinate - Optical Density

Abscissa - Wave Length, in Millimicrons

Heavy Line - Merck Corticosterone

Light Line - Biosynthetic Corticosterone

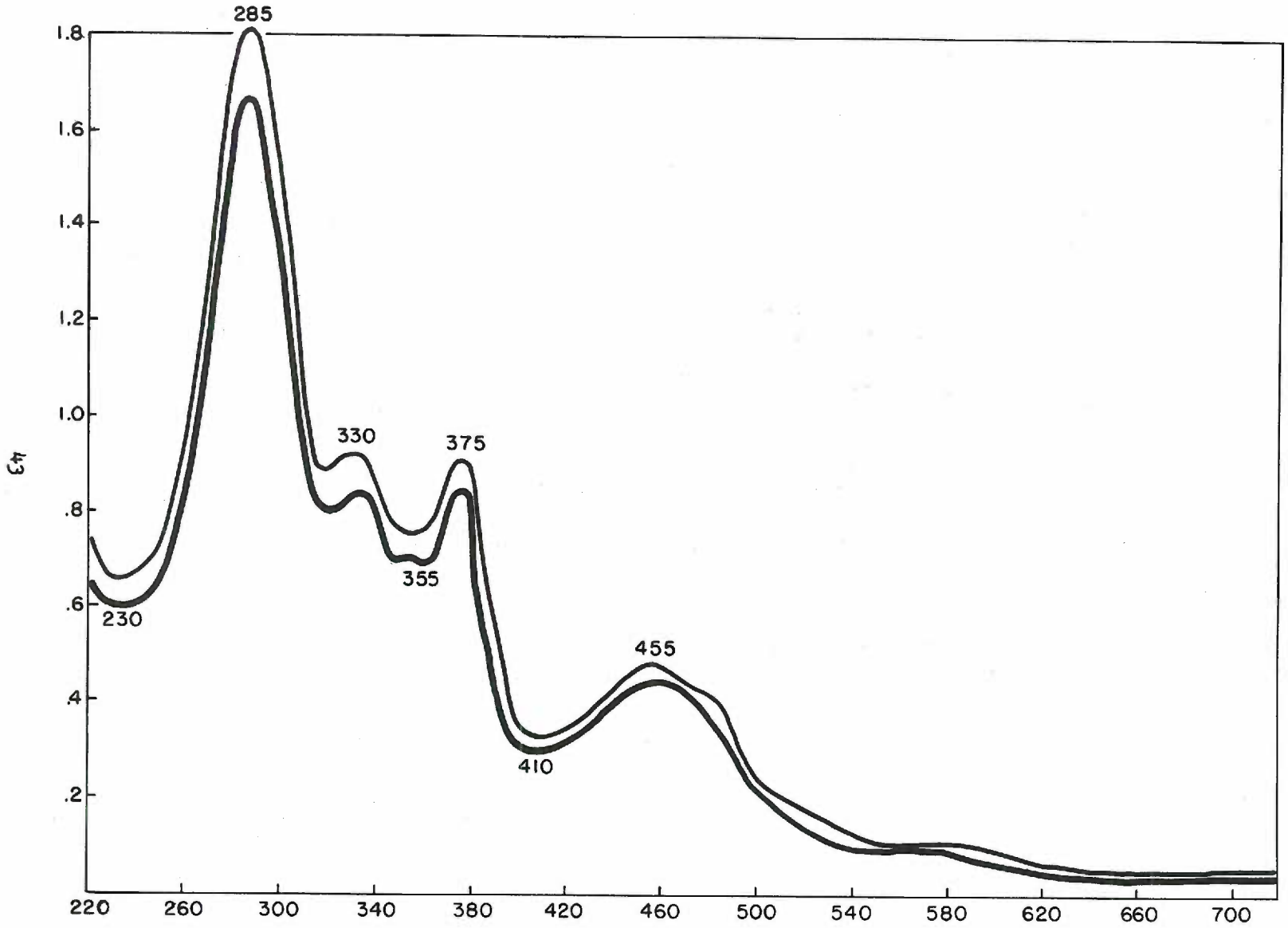

285
330
375
355
230
410
455
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
220
260
300
340
380
420
460
500
540
580
620
660
700

Figure 6

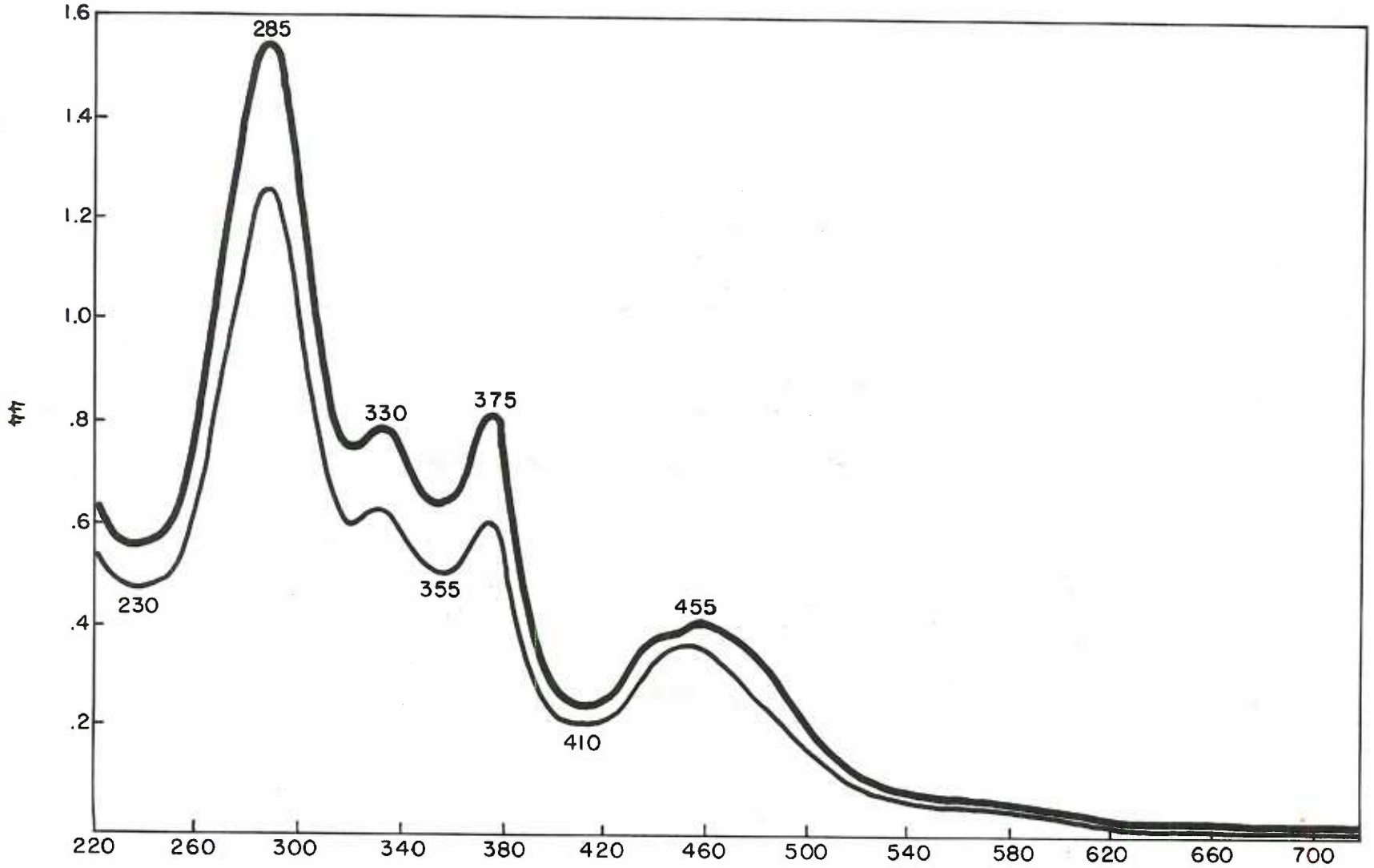
Ultra Violet Absorption Curve of a Pooled Sample from Five Incubations of Biosynthesized Corticosterone and an Authentic Sample of Corticosterone in Concentrated Sulfuric Acid

Note: Ordinate - Optical Density

Abscissa - Wave Length, in Millimicrons

Heavy Line - Merck Corticosterone

Light Line - Biosynthetic Corticosterone


1.6
1.4
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1.0
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700

comparison of this spectrum with those of the steroid samples is unnecessary since the double beam instrument used in this analysis compensates automatically for any water vapor present.

(5) All samples were paper chromatographed in a propylene glycol-toluene system using both ascending and descending techniques, simultaneously with the standard, as was a mixture containing all five samples, to determine both purity and homogeneity. The position of the steroid was located by staining with basic triphenyl tetrazolium blue, and by viewing under ultra violet light without staining. All sample spots, including that of the pooled sample, migrated uniformly to the same position as did the reference standard. There was no evidence of more than one spot produced by any of the samples. This speaks well for the homogeneity of the biosynthetic steroid. Typically, the spots in the ascending technique migrated to a position about one inch from the starting line, while those in the descending technique moved to a distance of approximately one and one-half to one and three-quarters inches from the point of origin.

C. O_2^{18} Uptake from H_2O^{18} Incubations

The remainder of the biosynthetic corticosterone was pooled into three samples for Unterzsucher pyrolysis and mass spectrography. The CO_2 obtained from pyrolyzing the

samples was analyzed spectrographically against a CO_2 standard. The expected atoms per cent O^{18} for a sample containing no atoms per cent excess O^{18} is 0.21%. All three samples were found to contain 0.21% atoms per cent O^{18}, the expected naturally occurring amount, which represents no uptake of O^{18} from the H_2O^{18} enriched water by the biosynthetic corticosterone.

The theoretical uptake for corticosterone synthesized from DOC in a system containing 1.4 atoms per cent excess O^{18} available would be 0.36 atoms per cent, or an atoms per cent excess of 0.36 - 0.21 = 0.15. Thus, one would expect that even if a small uptake of O_2^{18} from the solvent water had occurred, it would be quite apparent following mass spectrographic analysis, for if only a 20% theoretical uptake had occurred it would be reflected by an atoms per cent excess of 0.03, which in this range is significant. Table IV below is a report from Consolidated Electrodynamics Corporation of the mass spectrography of the derived CO_2.

TABLE IV

Mass Spectrometer Analyses

Sample No.	Ratio 46/44	Atoms % O^{18}	Atoms Excess % O^{18}
3268-9	.00417	0.21	.00
CO_2 Control	.00425	0.21	
3269-9	.00422	0.21	.00
CO_2 Control	.00424	0.21	
3270-9	.00419	0.21	.00
CO_2 Control	.00425	0.21	

D. Experiments with Catalase

The two original catalase experiments, along with the five modifications made upon the original method, gave consistently negative results, i.e., no conversion of DOC to corticosterone. Acid chromogen tests on (1) the direct chloroform extraction residue, (2) the residue after partitioning between 70% ethanol and hexane, and (3) the 5% ethanol in chloroform washes following column chromatography were all negative on all the incubations. For this reason no further tests were made, inasmuch as this test is sensitive enough to record quantities of corticosterone in the microgram range.

E. Experiments with Peroxidase

Eight incubations were made using horseradish peroxidase. The first of these was negative to an acid chromogen test on the same residue in the first modification of the original method, a duplicate run was made to isolate any biosynthesized corticosterone by subjecting the fat free residue to silica gel column chromatography. Results from the two 15 ml. 5% ethanol-in-chloroform washes from the silica gel column were negative. The five subsequent modifications also produced negative results by the acid chromogen test. Paper chromatography on a run involving modifications (1) through (4) revealed, however,

a spot occupying the same position as the spot of the authentic corticosterone control; however, methanol elution of this spot gave a negative test for corticosterone. A duplicate spot was eluted off and a sulfuric acid absorption spectrum taken. Figure 10 (page 52) is a graphic presentation of this spectrum. This spectrum was compared with those obtained by Zaffaroni[51,52] under similar conditions and resembled none of them. It exhibits an absorption maximum at 290 mμ which is typical of a steroid containing a Δ^4,3 keto grouping[52] and a small maximum at 490 mμ, which is atypical. Further, Zaffaroni states that compounds with only keto groups exhibit a single or no absorption maximum.

This steroid exhibited approximately the same polarity toward both paper and silica gel chromatography systems as did the reference corticosterone, appearing in the same eluent fraction following column chromatography and migrating to the same position in the propylene-glycol paper chromatography.

Further research into this particular section of the problem was not instigated as it represents, actually, a new problem.

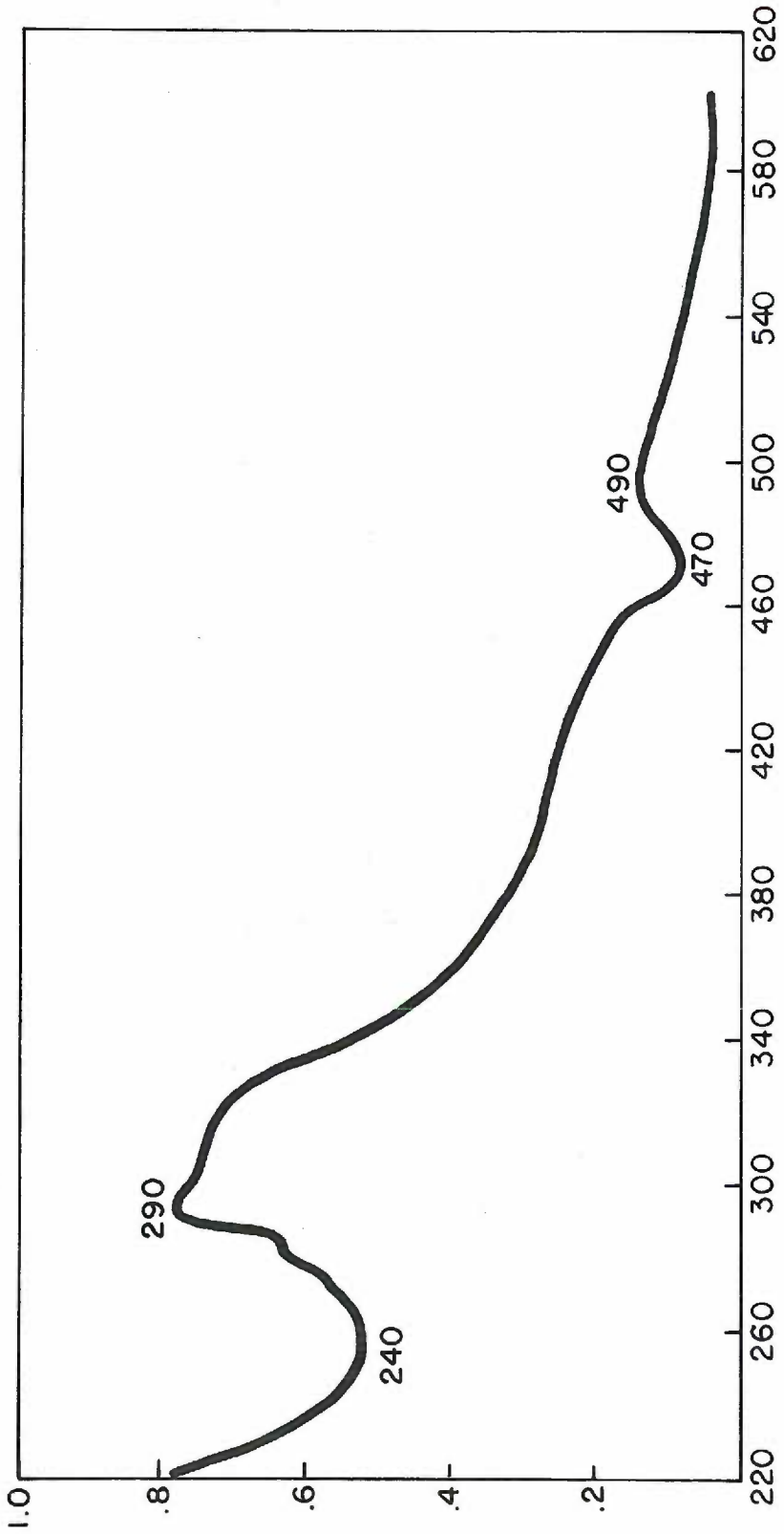
Figure 10

Ultra Violet Absorption Curve of Steroidal Substance
Isolated from Peroxidase Incubation

Read in Concentrated Sulfuric Acid

Note: Ordinate - Optical Density

Abscissa - Wave Length in Millimicrons


1.0
.8
.6
.4
.2
220
260
300
340
380
420
460
500
540
580
620
240
290
470
490

CHAPTER IV

DISCUSSION

A. Cofactor Requirements

(1) The Roles of TPN and Fumarate

Experiments using adrenal mitochondrial suspensions as catalysts of the 11-β hydroxylation of DOC by Hayano and Dorfman[16] led to their postulation that an absolute requirement of this system is fumarate. Brownie and Grant[17], however, were able to effect the same hydroxylation through the simultaneous addition of any one of a number of Krebs cycle acids besides fumarate, and further showed that the most powerful activation of this system occurred with the 'one step' oxidation of added α ketoglutarate. These workers explained this apparent discrepancy on the basis of a difference in the state of the mitochondria and claimed that the intensively washed mitochondrial preparations of Hayano and Dorfman exhibited a specific need for fumarate as a result of the loss of soluble enzymes active in the oxidation of other Krebs cycle intermediates. That such enzymes are localized in the mitochondria and such a loss does occur is shown in the work of Schneider[59] and Hogeboom and Kuff[60].

It would appear, then, that the observed cofactor requirements for the 11-β hydroxylation of DOC using adrenal mitochondrial suspensions as catalysts depend to a certain extent on how the tissue is prepared. In confirmation of this statement, Brownie and Grant[61], using deliberately damaged mitochondria, have been able to repeat the observation of Hayano and Dorfman[16] of the specificity of the fumarate requirement. Sweat[62] has observed that TPN is a necessary addition when aged or intensively washed tissue is used and further states that if fresh tissue is used the addition of exogenous TPN is unnecessary. This would bear out the studies of Siekevitz and Potter[63], who have shown that mitochondria contain constant amounts of bound nucleotides which are lost under a variety of conditions.

However, despite the different cofactor requirements noted with varying degrees of cellular alteration, and while these variances probably reflect an interdependency of cellular enzyme constituents, three observations of fundamental importance concerning 11-β hydroxylation have been described: Brownie and Grant[17] have shown that under no conditions will this reaction take place anaerobically; Sweat[18] has shown that reduced TPN in conjunction with a transhydrogenase enzyme is required; and Grant and Brownie[21] observed that reduced TPN in the presence of oxygen would permit 11-β hydroxylation in the absence of any Krebs cycle acids. These reports strongly suggest that the

function of fumarate or the other members of the Krebs cycle, known to catalyze this reaction, is the generation of reduced TPN. That this seems to be the case is borne out by the further findings of Grant and Brownie[81] in which they showed that in water soluble extracts of acetone dried mitochondria, fumarate was a specific cofactor requirement, as was TPN. The results of these investigators suggest that fumarate is required for the reduction of added TPN. These workers, in addition, produced evidence to show that the reduction of TPN proceeds through fumarate via malate by the intervention of Ochoa's malic enzyme[64], since pyruvate was isolated from the reaction mixture.

It thus appears that the two fundamental requirements for this enzyme system consist of oxygen and reduced TPN. The older view that concomitant oxidation of a Krebs cycle intermediate was necessary seems to be explainable now as a function in the reduction of TPN. In this sense, coupled oxidative phosphorylation would not appear to be a limiting factor. These findings are remarkably similar to those of Brodie et al[65] who, in a study of the detoxification of drugs by liver microsomes, noted the same requirement for TPNH and oxygen. This group postulated that a logical explanation would be that TPNH is oxidized by an oxidase system and the generated H_2O_2 subsequently utilized by a peroxidase type enzyme to accomplish the hydroxylation noted. Although coupled reactions of this type are fairly common[66],

Grant and Brownie[21] state that normally TPNH is oxidized in mitochondria via the flavoprotein cytochrome system with the production of H_2O and not H_2O_2. These workers caution that, although the production of H_2O_2 on TPNH oxidation may be a normal occurrence when the reduced flavoprotein formed reacts directly with oxygen in microsomal particles which are known to be deficient in cytochrome oxidase[67], such might not be the case in intact mitochondria.

Thus, while the available evidence strongly suggests that reduced TPN and oxygen are involved in a peroxidatic type of reaction, further work is necessary to justify this conclusion.

(2) The Role of Magnesium

The role of magnesium has not been as intensively studied as have the two cofactors previously discussed. Brownie and Grant[17] have shown the stimulating capacity which this substance has on 11-β hydroxylation but remain somewhat mystified as to its function, since ash analysis of adrenal mitochondrial preparations showed only 10 γ g. of magnesium oxide per two grams of tissue, a value which the workers considered low. Also, chelating agents such as ethylenediaminetetraacetic acid added to incubations had no effect. In a later study, Grant and Brownie[21] showed that in a more purified system a stimulating effect could be obtained through the use of manganese as well as magnesium.

Perhaps the report of Baltscheffsky[25] to the effect that magnesium functions in keeping the mitochondria in an intact morphological state might be of pertinent interest here.

B. Postulated Enzymic Mechanisms of 11-β Hydroxylation

(1) Experimental Evidence for and against

Mass spectrographic analyses on three pooled samples of corticosterone biosynthesized from 11-desoxycorticosterone in the presence of H_2O^{18} reveal that there was no incorporation of O^{18} into the 11-β hydroxyl group of the corticosterone. Results from a similar study in which the author participated, where the O^{18} was used as molecular oxygen, demonstrated conclusively that molecular oxygen but not water is utilized by the adrenal 11-β hydroxylating system in the C-11-β hydroxylation of steroids.

Accordingly, any attempt to explain the enzymic mechanism here involved not consistent with this finding sould be discarded. Further, any scheme postulated to explain the mechanism of action must take into account the fact that water is not utilized in the 11-β hydroxylation of steroids.

Several schemes have been offered by various authors to account for the mechanism of 11-β hydroxylation. Some are based on experimental evidence and others are purely conjectural in nature.

Scheme one[7] assumes the oxidation of the 11-desoxycorticosterone by an oxidase type of enzyme with the production of an 11-α hydroxy corticosterone, further oxidation to produce an 11 keto derivative, and reduction with inversion to form the 11-β hydroxy derivative. This scheme was ruled out by the observation[7] that the 11 keto steroid, 11-dehydro-17-hydroxycorticosterone, was not converted to 17-hydroxycorticosterone in either perfusions or homogenate incubations.

Scheme two would conceivably be the direct oxidation in the 11-β position by an oxidase type enzyme to produce corticosterone from 11-desoxycorticosterone. This hypothesis is unattractive from two standpoints. One is the fact that of the two hydrogens at C-11, the α hydrogen is reactive while the β hydrogen is chemically unreactive from a steric standpoint. Secondly, reduced TPN must also be accounted for in this scheme in conjunction with an oxidase type enzyme. This would necessitate the formation of a hydride ion in the activation of O_2 to form the reactive OH group; knowledge concerning the existence and function of hydride ions is at best nebulous. This criticism would apply to any oxidase type enzyme, keeping in mind the observed requirement for reduced TPN.

Mechanism three[6a] considers a system involving ring 'A' cleavage, allylic oxidation at the 'activated' C-11 position, and ring closure. No evidence for or against this

scheme has been offered, although the criticism of scheme two above would apply here.

Reaction sequence four, suggested by Levy et al[69], postulates the enzymic formation of free hydroxyl radicals which then react with 11-desoxycorticosterone to effect the direct oxidation to corticosterone. This sequence is definitely to be discarded on the basis of the experimental evidence presented in this thesis, which showed that there was no incorporation of O^{18} from the incubation H_2O^{18}. If a free radical mechanism were operating, one would expect, on the basis of exchange[70], to observe a small but significant incorporation from H_2O^{18}. Also, the yields in free radical reactions are usually low, whereas in the O_2^{18} studies yields of 85% of theoretical uptake occurred.

Sequence five is the proposed scheme of Hayano and Dorfman[20], in which an unsaturated 11-desoxycorticosterone derivative is an obligatory intermediate. These workers envisage a reaction whereby the addition of water to an unsaturated bond linkage would result in a hydroxylated product. This is analogous to the hydrase type of reaction seen in the Krebs cycle, in which fumarate is converted to malate. These workers tested this hypothesis using a Δ^{9-11} unsaturated 11-desoxycorticosterone and found that no conversion to corticosterone took place. However, the equally probable possibility that the $\Delta^{11,12}$ unsaturated analog of 11-DOC might serve as a substrate in this system was not

investigated. The results reported here of a complete lack of involvement of the solvent water in the transformation of DOC to corticosterone conclusively exclude this mechanism from consideration.

The remaining scheme, number six, is the possibility brought forth by Brodie[65] implicating a peroxidase as the enzyme system responsible for 11-β hydroxylation. From a consideration of the available data, this mechanism seems to be the most attractive. This hypothesis accounts for the observed necessity for reduced TPN; it provides for the requirement of oxygen; and it offers an explanation for the fact that water does not take part in the reaction.

However, while this latter scheme seems to fit the observed facts better than any of the preceding explanations, the cautionary note of Grant and Brownie[21] should be noted, i.e., that although this may be the case in mitochondrial preparations a different situation may exist in the completely intact cell.

(2) Experiments with Catalase and Peroxidase

Experiments in which crystalline beef liver catalase was used in an attempt to construct an artificial system capable of non-specific hydroxylations were completely negative. No explanation is offered for these negative results, although Keston and Carsiotis[71] have evidence to show the formation of more polar compounds than the

'parent compounds' in experiments in which iron, ascorbic acid, and oxygen served as a non-specific hydroxylating system for desoxycorticosterone acetate, corticosterone, and 11-dehydro-17-hydroxycorticosterone.

The steroidal substance obtained from incubations using horseradish peroxidase, dihydroxy fumaric acid, and oxygen, and DOC is most likely a poly hydroxy steroid, since it did not exhibit any of the tests for corticosterone but appeared in the same eluent fraction as corticosterone following column chromatography and migrated to the same position in both ascending and descending paper chromatography. These results are somewhat reminiscent of the work of Keilin and Hartree[78] who used catalase, peroxidase, and metmyoglobin as catalysts in coupled peroxidatic reactions.

Thus, although several schemes formulated to explain the phenomenon of 11-β hydroxylation have been offered and some of these disproved, the mechanism of 11-β hydroxylation remains obscure. Further investigation of the enzyme present in water soluble extracts of acetone dried mitochondria is indicated, as well as a careful consideration of the differences in requirements exhibited by isolated and 'intact' cell preparations.

CHAPTER V

SUMMARY

Desoxycorticosterone was incubated with adrenal mitochondrial preparations in the presence of H_2O^{18}. Mass spectrographic analyses of CO_2 derived from the pyrolysis of the biosynthesized corticosterone revealed that no incorporation of O^{18} from H_2O^{18} occurred. The pertinence of this finding has been discussed with respect to several postulated enzyme mechanisms.

Results from the incubation of catalase with DOC were negative with reference to the biosynthesis of corticosterone from such a system.

A steroidal substance similar to corticosterone in polarity was characterized in studies using peroxidase as a non-specific hydroxylating agent.

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APPENDIX

The author wishes at this point to include some additional acknowledgements and to cite additional literature which seems pertinent to this thesis.

At the time the work described herein was being done, other investigators were engaged in similar pursuits. Most notable among these were Hayano and Dorfman at the Worcester Foundation for Experimental Biology. These workers [73] demonstrated the lack of uptake of O^{18} from H_2O^{18} in incubations involving adrenal mitochondrial preparations with substrates including both 11-desoxycortisol and 11-desoxycorticosterone.

In a later report[74] this group showed in experiments involving microorganisms as a source of hydroxylating enzymes that 11β hydroxylation occurred in this case as did a variety of hydroxylations at other positions. That O_2^{18} is the source of oxygen for these hydroxylations was noted by these workers.

However, in view of the fact that a small but significant incorporation of O^{18} from H_2O^{18} by the substrate would throw much light on the enzyme process involved, the work already begun in this report was continued in the hope of perhaps demonstrating this small incorporation. No such incorporation did occur but definite proof of this is useful and has been discussed within the body of this thesis.

In addition, this writer apologizes for the regrettable omission of acknowledgements due to Dr. Max L. Sweat for constant advice and assistance and to Drs. Kerry Yasunobu and W. L. Fowlks who pyrrolyzed the steroid samples.

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Lastly, the author is indebted to Dr. H. S. Mason for the method utilized in the peroxidase experiments.

Important omissions from the literature include: (taken from Abstracts of Papers of the 130th meeting American Chemical Society Atlantic City, N. J., Sept. 16 to 21, 1956)

1. #124 p. 54c, Aromatic Hydroxylating Enzymes, Sidney Udenfriend, Chozo Mitoma and Herbert S. Posner.
2. #125 p. 55c, General Properties of Oxidases, H. S. Mason.
3. #126 p. 56c, Role of Oxygen and Reduced Pyridine Nucleotides in the Biogenesis of Cholesterol, T. T. Tchen and K. Bloch.
4. #128 p. 57c, Studies on the Mechanism of Steroid 11-β Hydroxylation, Gordon M. Tomkins.
5. #135 p. 60c, Hydroxylation, Peroxidase Activation of Oxygen, H. S. Mason.

and also:

6. Mason, H. S. and Onoprienko, I.; Mechanisms of Enzyme-Catalyzed Oxygen Transfer. Fed. Proc. 15:310, 1956.

In the light of the last two papers, in which it has been shown that (1) horseradish peroxidase catalyzes nonspecific aromatic hydroxylation in the presence of dihydroxyfumarate and molecular oxygen and (2) that the source of O_2 in the hydroxyl groups of salicylate incubated with the 'model hydroxylase', ferrous - EDTA - O_2 - ascorbate, is molecular oxygen; the scheme proposed by Dr. Mason for the hydroxylation reaction described in this thesis is most likely correct,

viz:

$$Fe_p^{++} + O_2 + 2e = Fe_p^{++}O + O^{--} \quad (1)$$

$$Fe_p^{++}O + AR\cdot H = Fe_p^{++} + AR\cdot OH \quad (2)$$

$$Fe_p^{++}O + 2e = Fe_p^{++} + O^{--} \quad (2a)$$

$$Fe_p^{++} + O_2 = Fe_p^{++}O_2 \quad (3)$$

with TPN·2H serving as the electron source.

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