STUDIES ON THE REGULATION OF 11β-HYDROXYLATION OF DEOXYCORTICOSTERONE AND OF PREGNENOLONE FORMATION IN ADRENAL MITOCHONDRIA

by

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Abstract

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The conversion of cholesterol to pregnenolone, the ACTH sensitive step of adrenal steroidogenesis, has been investigated with the aim of contributing to the understanding of the mechanism of action of ACTH. In order to carry out this study, an assay method for the determination of small amounts of pregnenolone, was developed. This assay method is based upon the enzymatic cycling of NADH produced by the oxidation of pregnenolone in the presence of 3β-hydroxysteroid dehydrogenase.

The kinetics of pregnenolone synthesis in vitro showed a biphasic time course; a fast primary rate followed by a slower secondary rate. In vitro ACTH treatment caused an increase in both rates, however the effect on the secondary rate was greater and the time required to establish this secondary rate was also increased by ACTH. Furthermore, the ACTH treatment resulted in an increase in

the mitochondrial pregnenolone steady state concentration when measured in intact mitochondria or in submitochondrial fractions. The inclusion of 20,22-dihydroxycholesterol (a precursor of pregnenolone) in the incubation medium caused a decrease in the time needed to attain the secondary rate. The above findings were not in accord with an originally adopted model in which ACTH was considered to bring about an increase in the rate of pregnenolone efflux from the mitochondria thereby relieving the feed-back inhibition by pregnenolone of its own synthesis. (A prediction of this model is that the stimulation of pregnenolone synthesis would be accompanied by a decrease in the intramitochondrial pregnenolone concentration.)

An alternate model which includes the above data proposes a modulation of the side-chain cleaving enzyme by ACTH, causing it to be less susceptible to an inhibitor, possibly pregnenolone.

In our continuing investigation of factors involved in the control of adrenal steroidogenesis it was found that ADP and ATP were inhibitory in a cell-free system for corticosterone synthesis from endogenous cholesterol. When each of the individual biosynthetic steps was investigated, the inhibition was found to be localized only at the 11β-hydroxylation of deoxycorticosterone (DOC) step in the mitochondria. The possibility that a control of steroidogenesis, secondary to ACTH action, is operating at that site led

to the further investigation of this inhibition. It was found that the inhibition required phosphate, was increased by the presence of glucose plus hexokinase, and was eliminated by the presence of atractyloside, phosphoenolpyruvate plus pyruvate kinase, or the substitution of isocitrate for succinate as the source of reducing equivalents. Therefore, it was concluded that ADP is the inhibitor and that the inhibition may be attributed to a competition for reducing equivalents between the hydroxylase systems and oxidative phosphorylation.

However, it was also observed that there was no inhibition if ATP was added 3 min. after the start of the incubation, and also that oligomycin did not completely reverse the ADP inhibition. These observations led us to suspect that ADP might inhibit the hydroxylation of DOC by means in addition to the competition previously mentioned. When disrupted adrenal mitochondria were used with NADPH as the source of reducing equivalents (under these conditions there is no energy required), Ca^{++} was found to stimulate the 11β-hydroxylase system (in conformation of the work of Péron) and ADP inhibited the Ca^{++} stimulation. The Ca^{++} stimulation was found to be very specific and Sr^{++} acted as a competitive inhibitor in this system. The kinetics of the system was studied; ADP was found to be a noncompetitive inhibitor with respect to Ca^{++}, Ca^{++} gave a so-called uncompetitive stimulation with respect to DOC, while ADP was

a noncompetitive inhibitor with respect to DOC. In the presence of 2mM deoxycholate, the Ca^{++} stimulation and the ADP inhibition were maintained. Furthermore, Ca^{++} and ADP did not have any effect on the binding of DOC to the mitochondria, as measured either by ^{3}H-DOC binding or by the induced type I difference spectrum. When the individual steps in the hydroxylating chain were investigated it was found that Ca^{++} and ADP had no effect on either the reduction of the flavoprotein or of adrenodoxin by NADPH; but it was also found that Ca^{++} stimulated the rate of cytochrome P450 reduction by NADPH and ADP inhibited that stimulation. It was concluded that the site of Ca^{++} activation and ADP inhibition is localized at the adrenodoxin-cytochrome P450 reduction steps possibly by modifying the interaction between these two proteins in the mitochondrial membrane.

FORWARD

The author wishes to express his sincere gratitude to Dr. Seymour B. Koritz for his guidance and courteous support throughout the course of this work.

Working in Dr. Koritz's laboratory was rewarding as a personal and intellectual experience.

The help I received from my lovely wife Zeinab through her understanding and patience was unlimited and the least I can do is to offer my sincere appreciation

LIST OF TABLES

LIST OF TABLES (Con't.)

LIST OF TABLES (Con't.)

Page No.

LIST OF FIGURES

TABLE OF CONTENTS

TABLE OF CONTENTS (Con't.) Page No.

TABLE OF CONTENTS (Con't.) Page No.

SECTION IV (Con't.)

LITERATURE REVIEW

Hydroxylation Reactions in Adrenal Steroid Metabolism.

The biosynthesis of corticosteroids in the adrenal gland involves a series of successive hydroxylations with the common requirement for NADPH and molecular oxygen. It has been demonstrated that one atom of each oxygen molecule is transferred to the substrate, while the other oxygen atom is reduced to water. Thus, the term "mixed function oxidase" was introduced by Mason (1) for hydroxylation reactions of this type, which is described by the general equation:

$$R\text{-}H + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+$$

The first indication for a possible role of a hemoprotein in steroid hydroxylations was presented by the work of Ryan and Engel (2). They observed that the microsomal 21-hydroxylation of 17-hydroxyprogesterone was inhibited by carbon monoxide and that the carbon monoxide inhibition was reversed by white light. Subsequently, a carbon monoxide binding pigment, with an intense absorption band at 450nm in the reduced state, was described by Klingenberg (3) in liver microsomes. This pigment was shown, by Omura and Sato (4,5), to be a hemoprotein possessing a protoheme as a prosthetic group and hence they introduced the term cytochrome P450 for this hemoprotein. Estabrook et al. (6) recognized the possible connection between the carbon monoxide inhibition of the 21-hydroxylase in adrenal microsomes and the carbon

monoxide binding pigment in liver microsomes. In a number of studies (6,7) they showed that bovine adrenal cortical microsomes contained a similar carbon monoxide binding pigment and they demonstrated the involvement of this pigment, as the oxygen activating component, in the 21-hydroxylation reaction. Cytochrome P450 has been identified also, by Harding et al. (8), in adrenal cortical mitochondria. Further studies by different investigating groups indicated the involvement of cytochrome P450 in drug metabolism in liver microsomes (see 9 for review), in steroid 11β-hydroxylation (10,11), in the side-chain cleavage of cholesterol (12) and steroid C-18 hydroxylation (13), in adrenal cortical mitochondria and in the C-17 and C-21 steroid hydroxylation in adrenal cortical microsomes (6). Omura and Sato (5) described the conversion of cytochrome P450 to a biologically inactive form by the use of detergents. This new form, when reduced, showed a maximum absorption band in the presence of carbon monoxide at 420nm and hence was given the term cytochrome P420.

The spectral properties of cytochrome P450 in liver microsomes were investigated by Remmer et al. (14) and Schenkman et al. (15). The addition of substrates to liver microsomal preparations resulted in certain specific spectral changes indicating that cytochrome P450 is the substrate binding species in the microsomal mixed function oxidation reactions. Two types of substrate induced difference

spectral changes were shown to occur in liver microsomes. The so-called type I difference spectra which was characterized by a trough at about 420nm, and a peak at 385nm, was induced by the addition of hexobarbital, phenobarbital and related substances to the microsomal preparations. Similar spectral changes were previously described by Narasimhulu et al.(16) upon the addition of 17-hydroxyprogesterone to adrenal microsomes. The other type of spectral change, type II, was characterized by a peak at about 430nm and a trough at 390nm and was induced by the addition of aniline and several other aromatic amines to liver microsomes. The work of Imai and Sato (17) indicated that the substrate induced spectral changes were the result of the substrate interaction with the oxidized form of cytochrome P450 and that change appears to be an obligatory step in the hydroxylation reactions. Studies carried out with other hemoproteins such as hemoglobin had shown that spectral changes similar to those seen in the above type I spectrum are strongly suggestive of a low spin (S=1/2)to a high spin (S=5/2) transition in the ferric iron of the hemoprotein. In contrast, a type II spectral change was found to be indicative of a high to low spin transition (18). Mason et al. (19) reported the presence of a unique electron paramagnetic resonance spectra (E.P.R.) for the low spin form of cytochrome P450. Whyener et al. (20) using sonicated adrenal mitochondria indicated that the type I response induced by the

addition of deoxycorticosterone (DOC), to these mitochondrial preparations, appears to be the result of a low spin to a high spin transition in the ferric iron of cytochrome P450 as seen by E.P.R. spectroscopy. Furthermore, they demonstrated that the type II spectral change was accompanied by an increase in the intensity of the low spin signals of the P450 cytochrome in these mitochondrial preparations. Thus, ligands interacting with oxidized P450 was classified as type I ligands which produce an absorption maximum at 390nm and form high spin substrate-enzyme complexes, and type II ligands which produce an absorption maximum at 420nm and form a low spin substrate-enzyme complex.

The studies on the spectral properties of cytochrome p450 at different stages of the hydroxylation cycle and also the substrate induced spectral changes of the oxidized P450, were found to be valuable not only in the understanding of the mechanism of steroid hydroxylation but also in the understanding of ACTH control of steroidogenesis (see below).

The Heterogenicity of Cytochrome P450 in the Adrenal Cortex.

The occurrence of more than one hydroxylation reaction in adrenal mitochondria or microsomes posed the question of whether different cytochromes P450 or only one cytochrome P450, with a multiple substrate specificity, are present in each of these organelles. Evidence for at least two different P450 cytochromes associated with adrenal cortical mi-

tochondria has been presented by various investigators. Clinical studies showed separate defects for each of the three mitochondrial mixed function oxidases (21). Spectral changes induced by selective steroids (20) and amines (22) suggested the presence of distinct P450 cytochromes in adrenal mitochondria. In addition, it was found that aminoglutethimide selectively inhibited the cholesterol side-chain cleavage (23) while metopirone selectively inhibited the 11β-hydroxylation of DOC (24). Finally, several investigators have successfully separated the cholesterol side-chain cleavage and the 11β-hydroxylase activities, free from one another, from beef adrenal cortical mitochondria (25-28).

Components of the P-450 Electron Transporting Chain.

The early work of Tomkins et al. (29,30) indicated the involvement of at least two protein fractions and an unknown heat-stable factor in the 11β-hydroxylation of DOC by adrenal mitochondria. Nakamura et al. (31) showed that this heat-stable factor was a protein and was further identified to be a non-heme iron protein which was given the name, adrenodoxin. The 11β-hydroxylase system was thus resolved into three specific and distinct proteins (33,34, 35), an FAD containing flavoprotein (adrenodoxin reductase or NADPH diaphorase), a non-heme iron protein (adrenodoxin or cytochrome P450 reductase) and a particulate fraction containing cytochrome P450. Omura et al. (33) showed that

the NADPH diaphorase isolated from microsomes could not substitute for the mitochondrial enzyme in a reconstituted system for 11β-hydroxylation. In addition, they found that, in contrast to the microsomal enzyme, the mitochondrial NADPH diaphorase was devoid of NADPH-cytochrome c reductase activity unless adrenodoxin was present. Recent immunological studies have shown that the mitochondrial NADPH diaphorase enzyme is also immunologically different from the microsomal enzyme in beef adrenal cortex (36). The cholesterol side-chain cleavage system of adrenal cortical mitochondria was subsequently resolved into three similar components by Simpson and Boyd (37) and by Bryson and Sweet (38).

Based upon spectral and electron paramagnetic resonance data, the present understanding (39) of the reaction sequences by which adrenal cortical mitochondrial and microsomal cytochrome P450, as the active center for steroid hydroxylation, interacts with substrates, oxygen and electron donors is presented in Fig. 1.

a) The binding of the substrate to oxidized cytochrome, ($P450\text{-}Fe^{3+}$), results in the formation of a high spin cytochrome P450-substrate complex ($\underset{|}{Fe^{3+}}\text{-}S$).

b) A single electron reduction of ($\underset{|}{Fe^{3+}}\text{-}S$) to the ferrous cytochrome P450-substrate complex ($\underset{|}{Fe^{2+}}\text{-}S$).

c) The formation of an oxygenated cytochrome P450-substrate complex ($\underset{|}{Fe^{2+}}\text{-}S$) upon the interaction of ($\underset{|}{Fe^{2+}}\text{-}S$) with oxygen. In the absence of oxygen, but in the

presence of carbon monoxide, (Fe^{2+}—S) will form a carbon monoxide complex characterized by a sharp absorption band at 450nm.

d) The above oxygenated complex ($Fe^{2+}_{O_2}$—S) is further reduced by a second single electron transfer. The exact species formed after this second reduction is not yet identified. Two possible structures were proposed for such an intermediate, a superoxide anion derivative of the ferrous cytochrome P450 complex or alternatively a hydroperoxide complex of the ferric form.

e) After the second reduction and by yet an unknown mechanism one oxygen atom is transferred to the substrate and the second oxygen atom is reduced to water.

f) The ferric form of the P450 (low spin) is regenerated upon the dissociation of the hydroxylated substrate.

As seen in Fig. 1 and previous discussion, the main difference between the adrenal mitochondrial and the adrenal or hepatic microsomal hydroxylation is the immediate source of electrons for the cytochrome P450 reduction. The studies of Omura et al. (33) and that of Kimura and Suzuki (35) indicated that adrenodoxin serves to mediate the oxidation of NADPH, via the flavoprotein, and the reduction of cytochrome P450 only in adrenal cortical mitochondria. Evidence for a role for adrenodoxin in addition to the simple reduction of cytochrome P450 is seen in the observations, by Kimura et al. (40), that reduction of P450

FIG. 1 Hypothetical mechanism of the steroid hydroxylation in adrenal cortical mitochondria and microsomes:

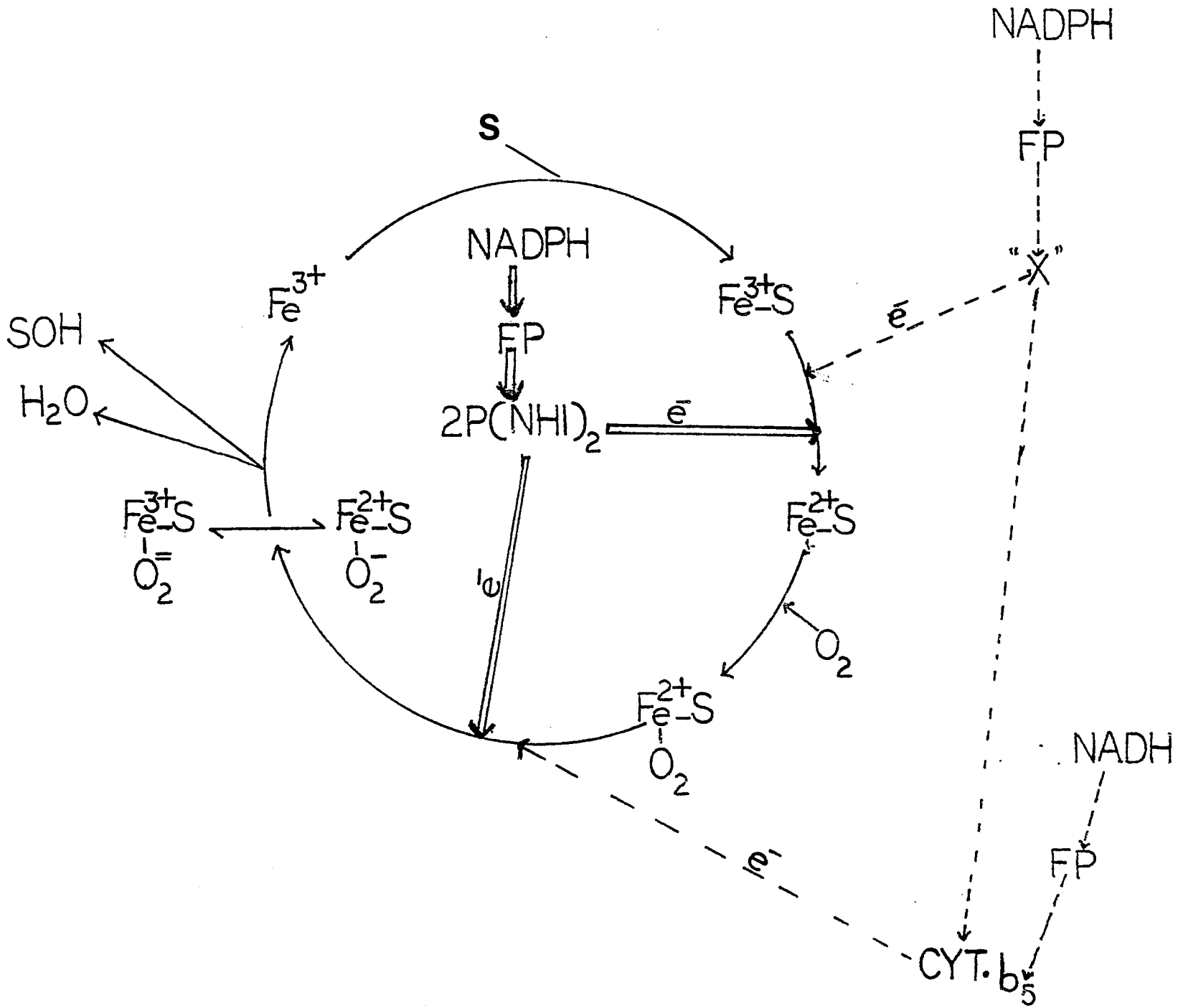
S = substrate

Mitochondrial electron flow double arrows:

FP = adrenodoxin reductase and
$P(NHI)_2$ = adrenodoxin

Microsomal electron flow dashed lines:

FP = the specific pyridine nucleotide dehydrogenase.
"X" = an unknown electron carrier and
Cyt-b_5 = cytochrome b_5


NADPH
FP
S
NADPH
FP
Fe^{3+}
$Fe^{3+}S$
SOH
H_2O
$2P(NHI)_2$
e^-
e^-
$Fe^{3+}S$
$O_2^=$
$Fe^{2+}S$
O_2^-
$Fe^{2+}S$
O_2
$Fe^{2+}S$
O_2
$2e^-$
NADH
FP
CYT. b_5

by artificial electron donors or by other non-heme iron proteins, i.e., reduced spinach ferridoxin, did not result in steroid hydroxylation. It appears that adrenodoxin must be the reducing agent for cytochrome P450 if steroid hydroxylation is to occur (40,41). As seen by the pathway given in Fig. 1,the reduction of cytochrome P450 is suggested to occur via two one-electron additions (40,41). The pathway of electron transfer from reduced pyridine nucleotide to the microsomal cytochrome P450 was studied mainly in liver microsomes. Orrenius (42) suggested that the NADPH diaphorase serves as the donor of the first electron for the cytochrome P450 reduction. Dallner et al. (43) indicated the possible mediation of an unknown electron transport component "X" between NADPH diaphorase and cytochrome P450. A possible role for cytochrome b_5 in the second reduction of the microsomal cytochrome P450 has been suggested by Hilderbrandt and Estabrook (44). Studies on drug hydroxylation in a reconstituted system from liver microsomes revealed the requirement for a heat-stable lipid fraction for these reactions (45). The active component of this lipid fraction was identified to be phosphotidylcholine by Strobel et al. (46). This phospholipid was shown to act on the cytochrome P450 reduction step (47).

The Source of Reducing Equivalents for the Adrenal Cortical Mitochondrial Hydroxylations.

It was observed, some years ago, that the steroid

11β-hydroxylation reaction could be supported by several Krebs-cycle intermediates (48-50). Both the 11β-hydroxylase (51) and the cholesterol side-chain cleavage enzymes (52) have an absolute requirement for NADPH and oxygen. Mitochondria are impermeable to pyridine nucleotides (53) therefore, NADPH required for these hydroxylations has to be generated within the mitochondria. Several pathways for the generation of mitochondrial NADPH from Krebs-cycle intermediates have been proposed. A possible pathway is the utilization of mitochondrial NADH, generated by the action of NAD-linked dehydrogenases, by an energy-linked NADH-NADP transhydrogenase activity. It was observed that both respiration and oxidative phosphorylation supported by NAD-linked substrates, were inhibited by the DOC hydroxylation in bovine (54) and rat (55) adrenal mitochondria. Such a competition for reducing equivalents suggested the involvement of a transhydrogenase reaction for the NADPH generation required for the 11β-hydroxylation. A number of other experimental findings support this conclusion and also indicate that the transhydrogenase is energy dependant. It was observed that the 11β-hydroxylation reaction, when supported by NAD-linked substrates, was inhibited by respiratory inhibitors (56,57) or by the uncoupling agent 2,4-dinitrophenol (56). It was also found that ATP reversed the inhibition of the 11β-hydroxylation seen in the presence of respiratory inhibitors (55). The presence of a

mitochondrial pyridine nucleotide transhydrogenase enzyme, in the adrenal, was established by the work of Stein et al. (58) and evidence for its participation in supporting the 11β-hydroxylase, in submitochondrial particles, was presented by Oldham et al. (59).

Harding et al. (57) showed that the 11β-hydroxylation, in rat adrenal mitochondria, was more effectively supported by succinate than by malate. The inhibition of the succinate-linked 11β-hydroxylation by amytal suggested to these investigators that reverse electron transport to generate intramitochondrial NADPH may be involved. Subsequently, Koritz (60) showed that succinate, to a greater extent than any other Krebs-cycle intermediate, supported the formation of pregnenolone from endogenous precursors, and this was also inhibited by amytal. Thus, reverse electron transport was implicated for generating NADPH needed for both the 11β-hydroxylase and the side-chain cleavage enzymes. NADPH may be also formed by the action of NADP-linked dehydrogenase in the mitochondria. Guerra et al. (56) and Sauer et al. (55) showed that the isocitrate-linked 11β-hydroxylation of DOC in rat adrenal mitochondria was insensitive to the presence of respiratory inhibitors. This was thought to be a reflection of the presence of NADP-linked isocitric dehydrogenase enzyme in these mitochondria. Another NADP-linked activity, the malic enzyme, has been found in beef adrenal cortical mitochondria, and was shown to be involved in

11β-hydroxylation, supported by malate in the presence of respiratory inhibitors (61). More recently, malic enzyme activity was also found in rat adrenal mitochondria (62).

As seen from the above discussion, the in vitro steroid hydroxylation may proceed by energy or nonenergy dependent paths, or by both, and with or without the involvement of transhydrogenase, depending on the substrate utilized. In addition to the apparent interaction between the respiratory chain and the hydroxylating chains in adrenal mitochondria, there is evidence also for a relationship between two of the hydroxylation reactions in these mitochondria. Such interaction was evident from the work of Yang and Hall (63) in which they showed that the side-chain cleavage of cholesterol was inhibited in the presence of DOC, but not vice versa. This inhibition was found to be partially, but not entirely, a result of a competition of the two hydroxylation reactions for NADPH.

The Side-Chain Cleavage of Cholesterol.

The enzymatic transformation of cholesterol to pregnenolone by the cholesterol side-chain cleavage enzyme complex has been presented as a consecutive irreversible reaction which involves the formation of hydroxylated intermediates [for review see (64)]. Until recently, evidence for the participation of a certain intermediate in the side-chain cleavage of cholesterol was based mainly on incubation stud-

ies in which a compound was considered to be a possible intermediate if it was metabolized to pregnenolone more readily than cholesterol. A number of hydroxylated cholesterol compounds were found to fulfill this criteria. It was found that (20S)-20-hydroxycholesterol, (20R,22R)-20,22-dihydroxycholesterol (65,66) and 22ξ-hydroxycholesterol (69) were transformed more efficiently, than cholesterol, to pregnenolone. Accordingly, two different pathways for the side-chain cleavage were proposed (68).

(1) Cholesterol ⟶ (20S)-20-hydroxycholesterol ⟶ (20R,22R)-20,22-dihydroxycholesterol ⟶ pregnenolone

(2) Cholesterol ⟶ 22ξ-hydroxycholesterol ⟶ (20R,22R)-20,22-dihydroxycholesterol ⟶ pregnenolone.

Burstein et al. (68) were able to establish the formation of (22R)-22-hydroxycholesterol and the (20R,22R)-20,22-dihydroxycholesterol when cholesterol was incubated with acetone-dried preparations of bovine adrenal cortex mitochondria. They also found that only exceedingly small amounts of (20S)-20-hydroxycholesterol could be found in such incubations. Thus, it was suggested by Burstein and Gut (64) that the side-chain cleavage of cholesterol is best described by sequence (2) with the isomer (22R)-22-hydroxycholesterol (69) being the first intermediate. However, they also indicated that assuming both sequence (1) and (2) are operating, only a relatively small fraction of the pregnenolone arising from cholesterol could be accounted

for. They offered two hypothetical explanations for this discrepancy.

a) The involvement of several one step or direct pathways, such as cholesterol ⟶ (20R,22R)-20,22-dihydroxycholesterol ⟶ pregnenolone or (22R)-22-hydroxycholesterol ⟶ pregnenolone. Therefore the rate of pregnenolone formation would be the sum of pregnenolone arising from these one step reactions plus the pregnenolone formed via sequence (2).

b) A relatively slow equilibration of the added hydroxylated cholesterol derivatives with enzyme complex as compared to a relatively fast conversion of these intermediates when formed *in situ*. In such a case the calculated conversion rates for these intermediates would be lower than their actual rate of conversion. Evidence for the existance of such slow equilibration in the acetone-dried powder preparations used in these studies has been presented by Burstein and Gut (70,71) who showed that the preincubation of the acetone-dried preparation with cholesterol or the hydroxylated intermediates resulted in an increase in the initial rate of product formation from these steroids.

Lieberman *et al.* (72) have suggested that the conversion of cholesterol to pregnenolone involve the formation of highly reactive intermediates in the form of free radicals or ionic species. In such a case, the hydroxylated choles-

terol compounds were considered not to be true intermediates and their formation was suggested to be a result of side reactions of these proposed highly reactive species. In support of this hypothesis, they found that the treatment of (20S)-20-hydroxycholesterol with lead tetracetate resulted in the formation of pregnenolone. This indicated that the side-chain cleavage of (20S)-20-hydroxycholesterol via the formation of free radicals is chemically feasible (72). The same group of investigators (73) demonstrated the enzymatic conversion of (20R)-20-t-butyl-5-pregnene-3β,20 diol, an analog for (20S)-20-hydroxycholesterol, to pregnenolone in the presence of sonicated bovine adrenal mitochondria. Since C-22 in this compound is completely substituted, therefore unavailable for hydroxylation, the involvement of transient free radicals (or ionic) intermediates was strongly implicated in the side-chain cleavage of this synthetic compound.

Recent mass spectrometric analysis of the enzymatic conversion of cholesterol to pregnenolone, in an $^{18}O_2$ enriched atmosphere revealed that the hydroxyl groups present on the side-chain of (22R)-22-hydroxycholesterol and (20R, 22R)-dihydroxycholesterol and also the C-20 oxygen in pregnenolone were all originated from molecular oxygen (74). The same investigators also showed that the two hydroxyl groups on the side-chain of (20R,22R)-20,22-dihydroxycholesterol were introduced by two different oxygen molecules. They interpreted this to indicate a sequential hydroxylation of cholesterol.

ACTH and Adrenal Steroidogenesis.

The biosynthesis of corticosteroids in the adrenal cortex consists of a series of reactions which occur in the mitochondria and the microsomes. Cholesterol was found to be the initial substrate (75). The side-chain cleavage of cholesterol to produce pregnenolone (52,76), steroid 11β-hydroxylase (76) and steroid 18-hydroxylase (13) activities were found to be localized in the mitochondria. Steroid C-21 (2) and C-17 hydroxylation (78) and the hydroxysteroid dehydrogenase/isomerase (79,80) activities were reported to be associated with the endoplasmic reticulum. The latter activity has also been reported to be present in the mitochondria (81).

Adrenal corticosteroid synthesis is stimulated by adrenocorticotropic hormone (ACTH). The first work on the site of action of ACTH in the steroidogenic sequence of reactions was carried out by Hechter et al. (82). These investigators showed that the production of corticosteroids in perfused adrenals is greatly enhanced by the presence of ACTH. To determine where the ACTH effect would be manifested, they perfused the adrenals with different steroid intermediates, in the presence and absence of ACTH. The results showed that ACTH stimulated steroidogenesis between cholesterol and progesterone. On the basis of kinetic evidence, Stone and Hechter (83) later concluded that the site of ACTH action is localized between cholesterol and

pregnenolone. This was confirmed by the work of Karaboyas and Koritz (84) who showed that ACTH enhanced the production of ^{14}C-corticosteroids from ^{14}C-cholesterol, in rat and beef adrenal slices, but had no effect on the ^{14}C-corticosteroid production from added ^{14}C-pregnenolone. Thus, the site of ACTH action was unequivocally shown to be localized at the cholesterol to pregnenolone step.

The initial report by Sutherland and Roll (85) in which they showed that cyclic 3'5' AMP (cAMP) mediated the epinephrine stimulation of glycogen phosphorylase in dog liver, led to the discovery by Haynes (86) that ACTH stimulated cAMP production in beef adrenal cortical slices. The first indication of the involvement of cAMP in steroidogenesis was presented by the study of Haynes et al. (87) in which they showed that cAMP, in the absence of ACTH, stimulated steroidogenesis in rat adrenal slices. Koritz (88) showed that the further addition of cAMP to maximally stimulated, by ACTH, rat adrenal slices resulted in no further increase in corticosteroid synthesis, indicating a common site of activation for both ACTH and cAMP. The above findings strongly suggested that the steroidogenic effect of ACTH is mediated via cAMP. This was strengthened by the finding that cAMP stimulated steroidogenesis at the same site as did ACTH (84), viz., between cholesterol and pregnenolone. Furthermore, it was found that ACTH increased cAMP levels, in rat adrenals, before the increase in steroidogenesis and

the magnitude of the increase in cAMP was a function of the amount of ACTH added (89). Lefkowitz et al. (90), using ^{125}I-labeled ACTH, showed that in a hormone sensitive plasma membrane preparation the ACTH was bound to these plasma membranes and activated the membrane bound adenyl cyclase enzyme, thereby stimulating the conversion of ATP to cAMP + PPi.

The possibility that the ACTH stimulation of steroidogenesis might involve the synthesis of protein(s) was considered a number of years ago. Experiments to test this possibility showed that ACTH had no effect on ^{14}C-glycine incorporation into proteins (91). In the presence of amino acid analogues it was found that, the steroidogenic response to ACTH was not affected in the face of 20-25% inhibition of protein synthesis. Thus, it was concluded that the action of ACTH did not involve an increase in protein synthesis (91). This conclusion was brought into question by the work of Ferguson (94) in which he used the more potent inhibitor of protein synthesis, puromycin, and showed that the presence of such an inhibitor abolished the steroidogenic response in adrenal slices to both ACTH and cAMP. Furthermore, Ferguson and Morita (93) reported that actinomycin D (an inhibitor of RNA synthesis) failed to inhibit the ACTH stimulation of steroidogenesis in the rat adrenal slices. Thus, it was concluded that protein synthesis but not RNA synthesis was required for the ACTH response. Garren et al. (94) extended this type of study

with a perfused rat adrenal gland and showed that both the onset and the maintenance of the ACTH steroidogenic response were sensitive to inhibitors of protein synthesis. The administration of puromycin to an ACTH stimulated gland resulted in a rapid decrease of the rate of steroidogenesis to the basal levels, indicating the involvement of a protein(s) with a rapid turnover. The half-life time of this protein was estimated to be in the order of 10 min. Therefore, these investigators suggested that ACTH steroidogenic response is mediated by initiating the synthesis of a rapidly turning-over protein (RT protein) and that the level of this RT protein determines the rate and the duration of the steroidogenic response.

However, it was argued that if the ACTH response is mediated by the initiation of protein synthesis, the newly synthesized RT protein would be more sensitive to protein inhibitors than the bulk proteins. This was found not to be the case in view of the reports by Hechter and Halkerston (95) and also by Kowal (96) and more recently by Koritz and Wiesner (97). In these studies it was found that inhibitors of protein synthesis decrease protein synthesis to a greater extent than they decrease ACTH or cAMP stimulated steroidogenesis. Furthermore, Koritz and Wiesner (97) showed that with low concentrations of inhibitors of protein synthesis, varying degrees of recovery of the rate of

steroidogenesis occurs with increasing time of incubation, while there was no effect on the inhibition of protein synthesis. These data suggest the accumulation of a limiting factor during the incubation period. Therefore, they suggested that the RT protein is normally present in excess amounts and that its rate of synthesis is independent of ACTH. ACTH, via cAMP, in such an interpretation was thought to act by transforming the RT protein to an active form, perhaps by phosphorylation, which in turn, stimulates pregnenolone synthesis. A built-in assumption for this model is that the half-life of the activated RT-protein must be assumed to be somewhat longer than that of the RT protein itself, inorder to permit its accumulation in the presence of protein inhibitors.

The accumulation of many new observation on the effects of ACTH on various aspects of adrenal steroidogenesis has led to a number of proposals to explain the mechanism of ACTH action.

1) Action via an increase in cytoplasmic NADPH.

Haynes and Berthet (98) showed the activation of glycogen phosphorylase by ACTH in bovine adrenal slices. This activation resulted in an increase in the level of glucose-6-phosphate which in turn can result in an increase in the formation of NADPH via the hexose monophosphate shunt. Thus, it was suggested by these investigators that ACTH stimulates steroidogenesis by increasing the production

of NADPH which is required for numerous hydroxylation reactions of steroidogenesis. However, this hypothesis was eventually rejected as a result of the large body of evidence which indicated that the effect of ACTH on the glycogen phosphorylase is superfluous to its action on steroidogenesis (92,99,100).

2) ACTH action via increased transport of cholesterol into the mitochondria.

A number of experimental findings, which were discussed previously, indicated that the ACTH steroidogenic response is mediated by cAMP. The understanding of the role of cAMP in steroidogenesis was enhanced by the finding that this cyclic nucleotide, which was previously shown to activate protein kinases in a number of mammalian tissues (101), activated the protein kinases in the adrenal gland. The mechanism of such an activation in the adrenal was found to be a result of cAMP binding to, and subsequently the dissociation of, an inhibitory (regulatory) subunit of the protein kinase (102). Furthermore, it was found that the cAMP dependent protein kinase was able to catalyze the phosphorylation of adrenal ribosomes in the presence of ATP. Thus, it was suggested that ACTH via the mediation of cAMP regulates, at the translation level, the synthesis of the RT protein. Another action of adrenal cAMP dependent protein kinase was the stimulation of adrenal cholesterol esterase (102,103). Accordingly, Garren et al.(102) pro-

posed a hypothesis for the ACTH action in which ACTH upon binding to the plasma membrane activates the adenyl cyclase to form cAMP. cAMP in turn activates the protein kinase which catalyzes the phosphorylation of ribosomes. This was thought to result in the initiation of the synthesis of the RT protein. The RT protein was then considered to participate in the transfer of free cholesterol, formed by the action of the protein kinase on cholesterol esterase, into the mitochondria and thereby providing the substrate for the rate-limiting step in steroidogenesis.

However, Mahaffee et al. (104) measured the cholesterol content in adrenal mitochondria obtained from hypophysectomized rats given ACTH alone or with cycloheximide. They observed that rats given ACTH alone showed a small increase in mitochondrial cholesterol while rats treated with cycloheximide plus ACTH showed a much greater increase in the mitochondrial cholesterol content. They concluded that the RT protein could not be involved in the transport of cholesterol into the mitochondria.

Furthermore, Mahaffee et al. (104) reported that the small increase in the mitochondrial cholesterol content found after ACTH administration could be markedly augmented if the animals also were given aminoglutethimide (an inhibitor of pregnenolone synthesis) 45 min. before the ACTH treatment.

When these mitochondria were used to study the in vitro

synthesis of pregnenolone, it was found that the ACTH treatment resulted in an increase in pregnenolone synthesis but more important, ACTH plus aminoglutethimide treatment resulted in a much greater increase in pregnenolone synthesis. Thus, these investigators suggested that ACTH stimulates steroidogenesis by regulating the precursor pool of free cholesterol that is available to the mitochondrial side-chain cleavage enzyme. Since they found that the accumulation of mitochondrial cholesterol was not sensitive to inhibitors of protein synthesis, they suggested that the RT protein must be involved in a later unknown step. The RT protein in such a hypothesis is thought not to be involved at the principal site of regulation. In a recent study Mahaffee and Ney (105) showed, using a sucrose gradient, the existance of two different populations of rat adrenal mitochondria, a heavy mitochondrial population and a light population with three times higher cholesterol content.

In addition, they found that prior treatment of the animal with aminoglutethimide plus ACTH resulted in an increase in the light mitochondrial population. This increase in mitochondrial cholesterol content was suggested to be the result of an increase in the degree, or the frequency, of the interaction between the mitochondria and fat droplets caused by ACTH. Such an interaction has been previously seen by electron microscopy by Idelman (106).

A similar model for ACTH action was proposed by a different group of investigators (107,108) and was based mainly on spectral evidence. They found that the addition of exogenous cholesterol or DOC to rat adrenal mitochondria resulted in induced type I spectral changes (high spin substrate complex) which were independent of one another. In these studies cholesterol was added in a lecithin emulsion. These results were interpreted by Harding et al. (108) to indicate a low level of endogenous cholesterol in these mitochondria. However, other investigators (109, 110) were unsuccessful in obtaining the same results when exogenous cholesterol was added in organic solvents. Harding et al. (107) also showed that the addition of electron donors, such as malate or isocitrate, to these mitochondria induced a type II spectral change (low spin). The magnitude of this spectral change was increased by the prior addition of exogenous cholesterol and was inhibited by aminoglutethimide. They interpreted this type II spectral change to be a result of the hydroxylation of cholesterol present in the mitochondria upon the addition of the electron donors. Other investigators (110) had found that pregnenolone also induces a type II spectrum and that this spectral change was correlated with a high spin cytochrome P450 complex detected by E.P.R. measurements (111). Harding (108) then studied isocitrate, cholesterol and pregnenolone induced spectral changes in adrenal mitochondria from rats

treated in various ways. It was found that ACTH treatment caused a small increase in the amount of cholesterol bound to P-450 and that this increase could be augmented by making the adrenals anoxic following the ACTH treatment. The anoxic treatment was suggested to block the hydroxylation of cholesterol resulting in an accumulation of mitochondrial cholesterol. Furthermore, it was found that the prior treatment of the rats with cycloheximide resulted also in an increase in the amount of the cholesterol bound to P-450. These results were interpreted to indicate that side-chain cleavage activity is regulated by the rate at which cholesterol is transported into the mitochondria and that ACTH treatment increases this rate. It was suggested by Harding et al. (107), in view of the results obtained from the cycloheximide treated animals, that the RT protein is involved at an unknown site in the hydroxylation sequence but not at the principal site of ACTH regulation. The kinetic parameters Km and Vmax for the side-chain cleavage enzyme was also studied, by Harding et al. (108), using ^{14}C-cholesterol and by measuring ^{14}C product formation. Essentially the same Km and Vmax values were found for the side-chain cleavage activity in mitochondria from hypophysectomized rats or from hypophysectomized rats given ACTH. This was interpreted to indicate that the ACTH treatment did not change the catalytic properties of this enzyme system.

However, the explanation of ACTH action only on the basis of an increase of cholesterol transport into the mitochondria suffers from the defect that it does not account for the sensitivity, of the ACTH steroidogenic response, to inhibitors of protein synthesis. Thus, the action of ACTH on the RT protein is still unresolved. Furthermore, it was observed that pregnenolone synthesis, from endogenous precursors, in isolated adrenal mitochondria could be stimulated under a variety of conditions, i.e., in the presence of Ca^{++} (112). This would indicate that the control of pregnenolone synthesis (the rate-limiting step) in these mitochondria depends on factors other than the mitochondrial cholesterol content. Finally, contrary findings have been reported from other laboratories with respect to the effect of cycloheximide treatment on the amount of mitochondrial cholesterol-bound to P-450 complex. On one hand, Harding et al. (107) reported an increase in the amount of this complex by the cycloheximide treatment while on the other hand, Brownie et al. (111) showed that the cycloheximide treatment blocked the ACTH induced increase of this mitochondrial P-450 cholesterol complex.

3) ACTH action via the modulation of side-chain cleavage enzyme activity.

Koritz and Kumar (113) first showed that adrenal mitochondria isolated from ACTH treated rats were several times more active in succinate-dependent pregnenolone formation

than mitochondria from control rats. They presented evidence that ACTH did not induce the formation of additional amounts of any of the enzymes involved in the conversion of cholesterol to corticosterone. Pregnenolone synthesis was found to proceed at a slower rate than any of the other reactions involved in the steroidogenic sequence. Furthermore, it was found that the corticosterone formation, in a system consisting of mitochondria, microsomes and other necessary factors, followed a biphasic time course, a fast primary rate followed by a slower secondary rate. ACTH treatment was found to increase both rates. Similar biphasic kinetics were observed when pregnenolone synthesis was measured directly by Simpson and Boyd (110) and more recently by Koritz and Moustafa (114). It has been shown previously that the in vitro rate of pregnenolone formation is enhanced under conditions in which the permeability properties of the mitochondria were changed (112). In addition, it was found that pregnenolone can control its own synthesis by inhibiting its formation from cholesterol (115) and this inhibition by pregnenolone was indicated to be allosteric in nature (116). Thus, it was postulated that ACTH, via cAMP, acts on the mitochondrial membranes to increase the rate of efflux of pregnenolone from the mitochondria. This will alleviate the pregnenolone feedback inhibition and will result in an overall increase in the rate of steroidogenesis (113). In view of such a model,

the fast primary rate seen in the in vitro synthesis of pregnenolone was interpreted to reflect a less inhibited synthesis resulting from the presence of an initial low level of mitochondrial pregnenolone. The secondary, slower rate, was suggested to be due to a higher steady state concentration of mitochondrial pregnenolone accumulated during the course of the primary rate. A critical prediction of this model is a decrease in the steady state concentration of mitochondrial pregnenolone following ACTH stimulation. This was recently tested and found not to be the case (114). Thus, the pregnenolone efflux model is not considered to be correct although control by an inhibitor may still be operative.

Alternative explanations for the mode of action for ACTH, based largely on spectral changes seen under various conditions in adrenal mitochondria, have come from the laboratories of Boyd and of Brownie. Jefcoate et al. (117) have studied the spectral changes induced by the addition of different steroids to adrenal mitochondria isolated from untreated, cycloheximide treated, and ether stressed rats. [It has been previously shown that 10 min. ether stress resulted in an acute increase in the blood level of ACTH and is thus equal to ACTH treatment (118)]. They found that the addition of DOC or 25α-hydroxycholesterol (the latter is a substrate for the side-chain cleavage enzyme) to rat adrenal mitochondria resulted in induced type I difference spectra. This spectral change was found to be independent of the pre-treatment of the

animals. On the other hand, they also found that the magnitude of the type II difference spectra induced by pregnenolone was larger with mitochondria isolated from stressed rats than from cycloheximide treated or untreated animals. This increase in the type II spectra was interpreted to indicate an increase in the amount of the high spin form of cholesterol bound-P450 complex induced by ACTH. It was also found that only the pregnenolone induced type II spectral change was sensitive to pH showing a maximum at lower pH values with a progressive decrease at higher pH values. The pH effect was found to be present regardless of the treatment of the animals. However, mitochondria from stressed rats showed type II change of greater magnitude than did the mitochondria from cycloheximide treated rats all through the pH range. Sonication of the mitochondria isolated from cycloheximide treated animals resulted in an increase in the type II spectral change, induced by pregnenolone, to a value equal to that obtained from intact or sonicated mitochondria from ether stressed rats. The sonication of the mitochondria also resulted in an increase in the biphasic rate of pregnenolone synthesis in mitochondria isolated from the three animal groups. Brownie et al. (119) showed that cycloheximide injection of rats maximally stimulated by ACTH caused a progressive decrease in the type II difference spectra obtained by the addition of pregnenolone to mitochondria isolated at different times after the cycloheximide treatment.

Thus, this spectral change [a measure of the high spin form of P-450 (111)] was correlated with the cycloheximide sensitive steroidogenic response.

The previous findings were interpreted by Jefcoate et al. (117) to indicate the presence of three forms of the cholesterol side-chain cleavage cytochrome P450 ($P450_{scc}$) in the isolated mitochondria.

a) a high spin $P450_{scc}$ cholesterol complex which is pH sensitive.

b) a low spin $P450_{scc}$ restricted from binding to cholesterol or to 25α-hydroxycholesterol and which is pH insensitive. The restriction to cholesterol binding could be removed and the pH sensitivity could be established by the prior treatment of the animals with ACTH or by sonication of the mitochondria.

c) a low spin form of $P450_{scc}$ which binds to 25α-hydroxycholesterol but not to cholesterol and which is pH and ACTH insensitive. Both pH sensitivity and cholesterol binding could be established by sonication of the mitochondria. Therefore, these investigators suggested a model for ACTH action in which ACTH removes, by some unknown mechanism, the restraint imposed on the $P450_{scc}$ described in (b) and they suggested a number of possibilities by which this restraint could occur. These possibilities include the presence of an endogenous inhibitor, or a change in the membrane environment, or alternatively, a

restriction on the mobility of cholesterol thus preventing its binding to cytochrome $P450_{scc}$. They feel that these possibilities can be encompassed in a model which centers upon the translocation of intramitochondrial cholesterol to $P450_{scc}$ (110,111,117). The *in vitro* biphasic rate curve of mitochondrial pregnenolone synthesis was interpreted to be the result of the presence of mitochondrial cholesterol in two pools. Cholesterol already bound to the $P450_{scc}$ gives rise to the first fast primary rate. The slower secondary rate is thought to reflect the rate of transport to or binding to the enzyme of cholesterol from a separate pool.

Brownie and Paul (120) and more recently Paul *et al.* (121) reported that warming up of mitochondrial preparations from 0°C was associated with the appearance of type I difference spectra. This indicated the formation of high spin cholesterol complex which was confirmed by E.P.R. measurement. The magnitude of this spectral change, which was determined "heat generated" type I spectral change, was dependent on the previous treatment of the animals from which the mitochondria was isolated. It was found that both ACTH and dibutyryl cAMP treatments resulted in an increase in the magnitude of this spectral change, while a prior treatment with cycloheximide prevented this ACTH induced increase. Brownie and Paul (121) suggested that the magnitude of the "heat generated" spectral change is determined by the level of some factor, possibly the labile (RT)

protein (102) which was found to be essential for ACTH action.

However, the interpretation of the steroid induced visible and E.P.R. spectral changes presented in the work from Boyd, Brownie and Harding laboratories (see above) was questioned by the finding that DOC addition to a P-450 preparation, active in both 11β-hydroxylation and side-chain cleavage, showed only a small change in both visible and E.P.R. spectra. Therefore, it was concluded by Schleyer et al. (122) that the change in spin state equilibrium of cytochrome P450 is not an obligatory part in the reaction mechanism of steroid hydroxylation. Furthermore, Burstein et al. (123), using acetone-dried preparations of adrenal mitochondria, found no correlation between the side-chain cleavage activity and the spectral difference induced by the addition of several hydroxylated cholesterol to these compounds.

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SECTION I - SOME CHARACTERISTICS OF ADRENAL STEROIDOGENESIS AND THEIR POSSIBLE RELATIONSHIPS TO THE ACTION OF THE ADRENOCORTICOTROPIC HORMONE

INTRODUCTION

The acute affect of ACTH on adrenal steroidogenesis resides in the stimulation of the rate of conversion of cholesterol to pregnenolone (1-4). This reaction sequence occurs in the mitochondria (5) and it has been shown that pregnenolone synthesis in this organelle is increased after the administration of ACTH *in vivo* (6,7). Indeed, the conversion of cholesterol to pregnenolone, of all the reactions involved in the transformation of cholesterol to corticosterone, is the only step affected by ACTH (7).

Additional data concerning this transformation have been obtained by investigating the kinetics of corticosterone synthesis in a cell-free system from rat adrenals (7). The observed rate of corticosterone synthesis at 20°C was biphasic with a rapid primary rate lasting for 4 to 6 min., followed by a slower secondary rate which continues for at least another 15 min. The formation of pregnenolone was shown to be the rate-limiting step in corticosterone synthesis, even after ACTH stimulation. Hence, it was concluded that the biphasic rate curve for corticosterone synthesis is a reflection of this step in the reaction sequence. Other investigators also have obtained a biphasic rate curve when directly measuring pregnenolone synthesis in isolated adrenal mitochondria (8,9). Since both rates are increased by the prior administration of ACTH *in vivo*, an explanation of this biphasic curve may lead to a greater understanding

of the mechanism by which ACTH controls steroidogenesis.

The primary aim of the present study was to define clearly in quantitative terms the characteristics of the biphasic rate curve for pregnenolone synthesis in cell-free systems from the adrenal using both control and ACTH-treated rats. The data obtained have provided additional guidelines for any model describing ACTH action, and have been used in an attempt to test the adequacy of some current models for ACTH action based upon this biphasic curve.

MATERIALS AND METHODS

Preparation of the mitochondria and the microsomes: Male Sprague-Dawley rats weighing 175-210g were killed by decapitation. When ACTH was used, 2 units in 0.2ml of 0.9% sodium chloride were injected intraperitoneally 15 min. before killing. The preparation of the tissue has been described previously (7). The subcellular fractions were prepared in the following manner. A 10% homogenate of the rat adrenals was made in 12mM Tris, pH7.5, 1mM EDTA and 0.25M sucrose with 3-5 strokes of a motor-driven Teflon pestle. The homogenate was centrifuged at 440 x g for 10 min. and the pellet discarded. (The relative centrifugal force was calculated at the maximum radius of centrifugation.) The supernatant was diluted 2-3 fold with the homogenization medium and centrifuged at 9,000 x g for 10 min. The mitochondrial pellet so obtained was resuspended in the homogenization medium to the diluted volume and centrifuged at

4,900 x g for 10 min. The resulting pellet was washed a total of 3 times by this procedure and finally suspended in the homogenization medium to the original volume of the homogenate. Microsomes were obtained from the supernatant from the first mitochondrial pellet (7).

Incubation conditions for corticosterone and pregnenolone synthesis: Incubation conditions and the fluorometric analysis for corticosterone synthesis were carried out as previously described (7) except in those incubations which involved 20,22-dihydroxycholesterol where 0.2ml of 10% bovine serum albumin per 2.5 ml of incubation medium was added. Incubations for pregnenolone synthesis from endogenous precursors were carried out in 20ml beakers at 20°C in a Dubnoff Metabolic Incubator. The complete system consisted of 0.4ml of mitochondria, 0.3ml of 0.3M Tris buffer at pH7.5, 0.15ml of 0.2M potassium phosphate at pH7.5, 0.15ml of 0.1M $MgCl_2$, 0.15ml of 0.2M potassium succinate at pH7.5, 0.2ml of 10% bovine serum albumin, 0.04ml of ethanol, 0.1ml of alcohol dehydrogenase containing 12 units of activity, and the homogenization medium to a final volume of 2.5ml. Aliquots of 0.4ml to 1.0ml were removed at indicated times and delivered into glass-stoppered tubes containing 3ml of dichloromethane. After shaking and centrifugation a 1- or 2ml aliquot of the dichloromethane was removed and analyzed for pregnenolone. Zero time controls without succinate and Mg^{++} were routinely carried

out. The inclusion of bovine serum albumin, ethanol and alcohol dehydrogenase in the incubation medium was based on the following findings and considerations. Incubation of pregnenolone with mitochondria for 20 min. at 20°C resulted in a 70%, or greater, disappearance of the pregnenolone. This loss is eliminated if the mitochondria are boiled, indicating an enzymatic process. This is probably due to the transformation to progesterone by steroid dehydrogenase, since the addition of ethanol plus alcohol dehydrogenase to reduce endogenous NAD^+ increased the recovery. The further addition of bovine serum albumin, which probably acts by binding the pregnenolone, resulted in a recovery of 85%-95% of the added pregnenolone. Cyanoketone could not be used since it interferes with the assay method.

To determine the distribution of pregnenolone between the mitochondrial pellet and the supernatant during an incubation, a 1ml aliquot of the entire medium was taken for pregnenolone assay and another 1ml aliquot was transferred to a 1.5ml plastic centrifuge tube in an ice-bath. At the conclusion of the incubation the tubes were centrifuged at 20,000 x g for 5 min., and the supernatants and mitochondrial pellets (removed with two 0.5ml portions of 0.25M sucrose) extracted with dichloromethane.

Pregnenolone determinations: The determination of pregnenolone was based upon the enzymatic cycling of the NADH produced by the oxidation of pregnenolone in the presence of

β-hydroxysteroid dehydrogenase and the fluorometric determination of the augmented NADH produced. Since the original description by Lowry et al.(10) of the measurement of pyridine nucleotides by enzymatic cycling, a number of modifications and applications to various substances have appeared. The present procedure is a modification of one described by Anggard et al.(11) for the determination of prostaglandins.

Reagents. Reagent I - To a 10ml volumetric flask add 0.1ml of a freshly prepared 1.2% (v/v) solution of mercaptoethanol, 1.5mg of NADand 0.25ml of a 1 to 100 dilution of β-hydroxysteroid dehydrogenase. The volume is made up to 10ml with 0.1M Tris buffer, pH9. The final concentrations are 2mM mercaptoethanol and 0.2mM NAD.

Reagent II - To a 10ml volumetric flask add 0.05ml of a 0.62% (w/v) solution of sodium arsenate (0.1mM final concentration), the equivalent of 0.17ml of the free acid of DL-glyceraldehyde-3-phosphate neutralized to pH5.2 with NaOH (2.5mM), 3.6mg α-ketoglutaric acid (2.5mM), 1.5mg ADP (0.3mM), 1ml of a 4.16% solution of tetrasodium EDTA (10mM), 0.25ml of a freshly prepared 1.2% (v/v) solution of mercaptoethanol (5mM), 0.5ml of a 0.77% (w/v) solution of ammonium acetate (5mM), 0.35ml of the Norit treated glutamic dehydrogenase and 0.35ml of the Norit treated glyceraldehyde-3-phosphate dehydrogenase. The volume is made up to 10ml with water. The concentrations given in parenthesis refer to the final concentrations present in this

solution.

Reagent III - To a 10ml volumetric flask add 11.2mg NAD (1.5mM), 3.7mg ADP (0.75mM), 5.0ml of a 6.7% solution of semicarbazide hydrochloride adjusted to pH9.5 with NaOH (0.3mM) and 50µl of glutamic dehydrogenase (not Norit treated). The volume was made up to 10ml with 0.4M Tris Buffer, pH9.5.

Procedure. To 50µl of reagent I add 5µl of the sample dissolved in dioxane and let the mixture incubate for 40 min. at room temperature. Add 5µl of 0.8 N NaOH, mix thoroughly, and heat at 60°C for 20 min. Cool the tubes and add 500µl of reagent II. After cycling for 60 min. at 37°C, 5µl of 4 N HCl containing 0.75% H_2O_2 is added and the tubes placed in a boiling water bath for 2 minutes. Cool the tubes and add 1.0ml of reagent III and incubate at room temperature for 60 min. The fluorescence is read in 75 x 10mm tubes in a Turner model 111 fluorometer using primary filter 6-70 and secondary filter 48 plus 2A. It is seen from Figure 1 that a linear relation exists between the fluorescence and the amount of added NADH or pregnenolone. The equivalent fluorescence on a molar basis between pregnenolone and NADH indicates that the pregnenolone added is completely oxidized.

Recovery experiments were carried out under conditions in which different amounts of pregnenolone were added to mock incubation mixtures with and without rat adrenal mitochondria. The mixtures were extracted with dichloromethane,

FIG. 2. Relationship between fluorescence and the amount of NADH or pregnenolone. o-NADH; ●-pregnenolone.

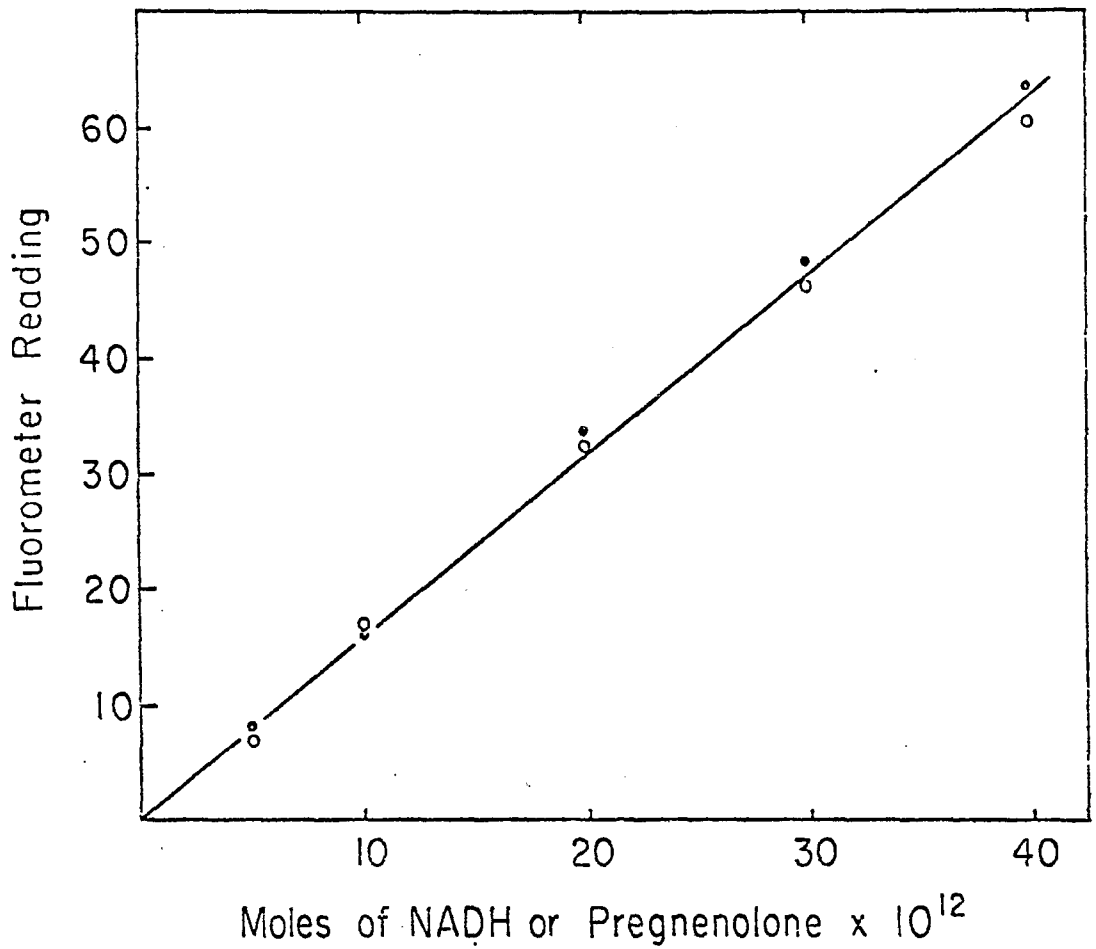
Fluorometer Reading
60
50
40
30
20
10
10
20
30
40
Moles of NADH or Pregnenolone x 10^{12}

TABLE 1

The Recovery of Pregnenolone in the Presence and Absence of Rat Adrenal Mitochondria

Pregnenolone Added	Fluorometer Reading	
	Mitochondria	
Moles x 10^{12}	Absent	Present
25	36	36
37	56	55
50	70	70

the dichloromethane evaporated under nitrogen and the residue taken up in 5µl of dioxane for analysis. The results of such an experiment are given in Table 1. It is seen that the presence of mitochondria did not reduce the recovery of pregnenolone. In addition, a comparison of the fluorometer readings with those obtained in the determination of the standard curve (Figure 2) indicates that no loss of pregnenolone occurred in the various manipulations involved in this experiment.

Basically, the specificity of this and similar methods depends on the enzyme preparation involved in the initial formation of NADH from the substance to be determined (reagent I). In the present case the preparation of 3β-hydroxysteroid dehydrogenase used is only partially purified and contains, for example, some alcohol dehydrogenase so that the use of ethanol is to be avoided in this step. An additional measure of specificity, is introduced by the fact that the determination of pregnenolone involves an initial dichloromethane, or other organic solvent, extraction of the sample to be analyzed so that water soluble substances are excluded. The steroids commonly found in the adrenal were tested at the 2µg level. Corticosterone, cortisol, cortisone, 11-deoxycorticosterone and progesterone were completely negative. Cholesterol obtained from Mann Biochemicals gave a fluorometer reading in the assay 300 times less than an equimolar amount of pregnenolone. Samples of cholesterol from different sources

gave different values which suggests that the positive readings obtained in the assay may be due to contaminants in the cholesterol. Furthermore, excellent agreement was obtained, for the determination of pregnenolone, between the previously described method and the colorimetric (8) method of lower sensitivity but which has been shown by chromatographic verification to specifically measure pregnenolone.

In the experiments presented in Tables 4 and 5 the determination of the small amounts of pregnenolone present required the use of a radioimmuno-assay. The dichloromethane extracts of the experimental material was evaporated and the residue subjected to Sephadex LH-20 chromatography as described by Bartosik and Szaeowski (12). The pregnenolone peak was collected, evaporated to dryness and taken up in methanol. An aliquot was assayed directly for progesterone by a radioimmuno-assay (13) and another aliquot evaporated to dryness, taken up in dioxane and treated with 0.5ml of reagent I. The mixture was extracted with dichloromethane, taken to dryness, the residue dissolved in methanol and assayed for progesterone. The two assays permitted the determination of the pregnenolone present in the original sample taking into account recoveries as determined by tracer 7α-^{3}H-pregnenolone initially added.

The author is indebted to Dr. Delphine Bartosik for her guidance and the use of her laboratory at the New York Medical College in carrying out the radioimmuno-assays.

Materials: The alcohol dehydrogenase from yeast was a crystallized powder which contained 200 units per mg protein. The bovine serum albumin was a crystallized, lyophilized preparation. Both products were obtained from Sigma Chemical Company. The active isomer (20R,22R)-20,22-dihydroxycholesterol, was a gift from Dr. S. Burstein. Mercaptoethanol and semicarbazide HCl were obtained from J.T. Baker Company. NAD·3.5 H_2O, Dl-glyceraldehyde-3-phosphate as a solution of the free acid (50mg/ml), Na_2NADH·1.5 H_2O, NaADP·2 H_2O, Na_4 ethylenediamine tetracetate·2H_2O (EDTA), and Tris (hydroxymethyl) aminomethane (Tris) were obtained from the Sigma Chemical Company. Sodium arsenate·7 H_2O was obtained from Fisher, ammonium acetate from Mallinckrot and α-ketoglutaric acid from the California Corporation for Biochemical Research.

Glutamic dehydrogenase as a solution in 50% glycerol and with a specific activity of approximately 45μ/mg and glyceraldehyde-3-phosphate dehydrogenase as a crystalline suspension in 0.25M ammonium sulfate and with a specific activity of approximately 36μ/mg were obtained from Boehringer. To remove endogenous pyridine nucleotides these two enzymes were treated with charcoal (Norit). Neutral Norit was washed several times with 0.05 M Tris acetate-0.002 M EDTA buffer, pH7.5, and then dried with ethanol and ether. To 0.9ml of cold Tris-EDTA buffer 0.1ml of the enzyme and 10mg of Norit were added and the mixture gently shaken. After 10 min. on ice it was centri-

fuged in the cold at 6,600 x g for 3 min. and the supernatant passed through a sintered glass filter. Without such treatment use of these enzymes in reagent II resulted in unacceptably high blank values.

3β-Hydroxysteroid dehydrogenase was assayed and prepared according to the procedures described by Talalay (14). Purification was carried out through the ammonium sulfate fractionation steps and the preparation obtained had a specific activity, with testesterone as the substrate, of 25,000 units per mg protein. This value was obtained with turbidimetric method for the protein determination as used by Talalay. However, with a biuret method for the protein the specific activity was 8,000 units per mg protein. The enzyme preparation was stored in 5ml portions at -20° C.

The progesterone antibody used in the radioimmuno-assay was a gift from Dr. Delphine Bartosik.

RESULTS

In the first experiments the kinetics of pregnenolone synthesis in mitochondria from adrenals from control and ACTH-treated rats were determined. Biphasic rate curves were obtained as anticipated by previous studies of corticosterone synthesis (7) and in confirmation of the results of Simpson *et al.* (8). Figure 3 presents a typical time course of pregnenolone synthesis in adrenal mitochondria, in this case from control rats. It is seen that a well defined transition from the primary rate to the secondary rate is present. Routinely, this point was determined by the intersection between the two rates. The data in Table 2 show that ACTH administration *in vivo* results in a larger percentage of stimulation in the secondary rate of pregnenolone synthesis than in the primary rate, concomitant with an increase in the duration of the primary rate. Similar results were obtained when the complete steroidogenic sequence of reactions to form corticosterone was measured in a cell-free system (Table 3). The detailed time course of corticosterone synthesis has been previously presented (7) and is also given in Fig. 4.

It has been proposed that ACTH may act by increasing the rate of efflux of pregnenolone from the mitochondria, thereby relieving an inhibition of the conversion of the cholesterol to pregnenolone. Such a model predicts that a steady state concentration of pregnenolone would be pre-

FIG. 3. The time-course of synthesis of pregnenolone from endogenous precursors in adrenal mitochondria from control rats. Incubation conditions are given in the text. Each time point was determined in triplicate and the extremes plus the mean for each time point are indicated.

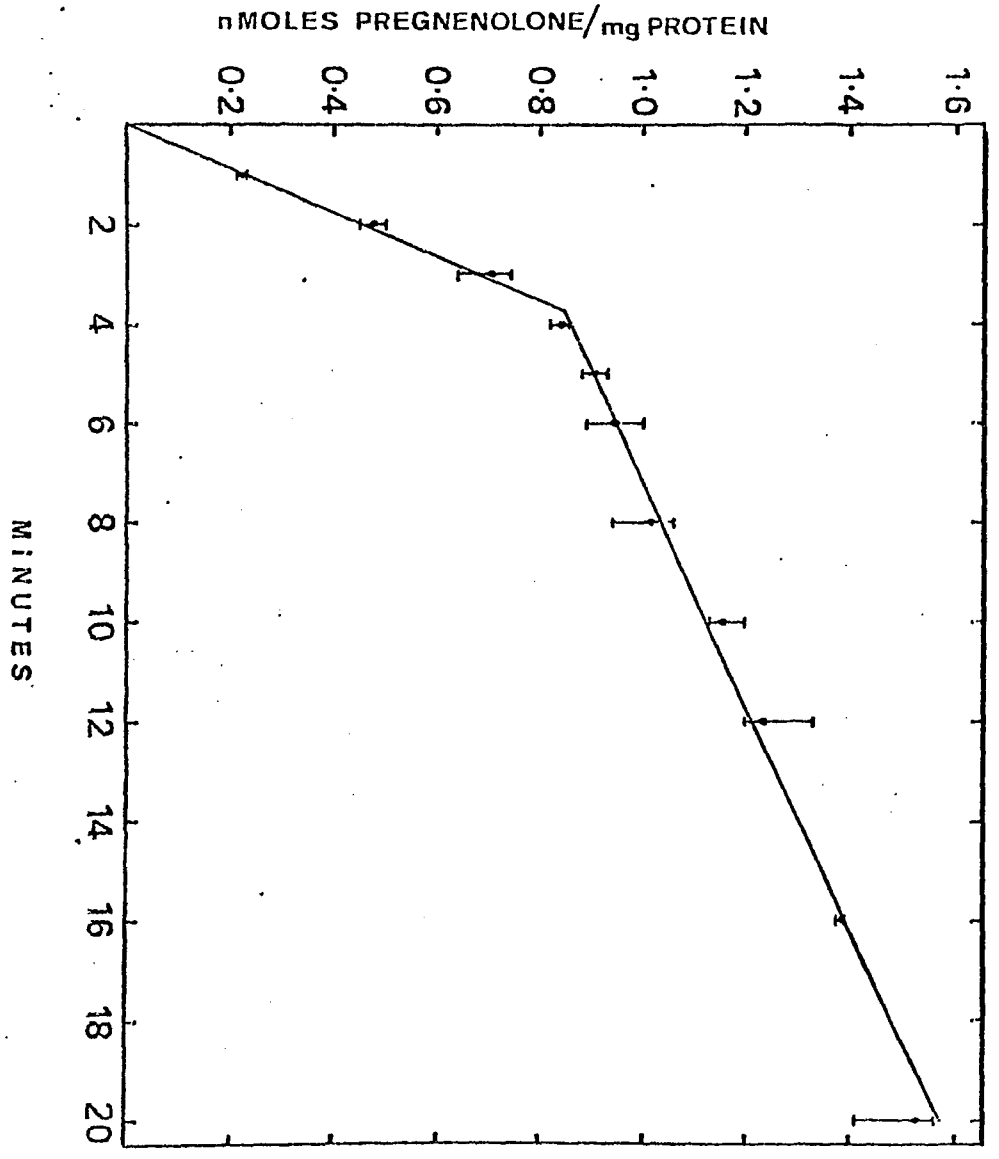
nMOLES PREGNENOLONE/mg PROTEIN
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
MINUTES
2
4
6
8
10
12
14
16
18
20

TABLE 2 - The course of pregnenolone synthesis in adrenal mitochondria from control rats and rats given ACTH. Aliquots were taken at 1.5,3,6,10 and 14 min. to give at least two time points plus the zero time to determine primary rate and two to three time points to determine the secondary rate.

The data are given ±S.E. and represent 12 experiments with mitochondria from control rats and 10 experiments with mitochondria from ACTH-treated rats. The supernatant is that obtained following centrifugation of the entire incubation medium.

TABLE 2

The Course of Pregnenolone Synthesis in Adrenal Mitochondria from Control Rats and Rats given ACTH

	Control	ACTH	Change	p Value
	nmoles/min/mg protein		%	
Entire medium				
Primary rate	0.24 ± 0.02	0.33 ± 0.03	+ 37	.020-.025
Secondary rate	0.045 ± 0.005	0.11 ± 0.01	+145	<.001
	minutes			
Duration of primary rate	4.0 ± 0.2	6.9 ± 0.5	+ 72	<.001
	nmoles/min/mg protein			
Medium supernatant				
Primary rate	0.16 ± 0.015	0.23 ± 0.02	+ 43	.010-.020
Secondary rate	0.038 ± 0.005	0.095 ± 0.01	+150	<.001
	minutes			
Duration of primary rate	3.7 ± 0.2	7.0 ± 0.6	+ 89	<.001
	nmoles/mg protein			
Pregnenolone in the Mitochondrial pellet	0.29 ± 0.03	0.37 ± 0.02	+ 27	.05-.10

TABLE 3

The Course of Corticosterone Synthesis in an Adrenal Cell-Free System from Control Rats and Rats given ACTH

	Control	ACTH	Change %	p Value
	nmoles/min/mg mitochondrial protein			
Primary rate	0.30 ± 0.02	0.58 ± 0.05	+ 94	<0.001
Secondary rate	0.052 ± 0.003	0.13 ± 0.01	+150	<0.001
	minutes			
Duration of primary rate	5.5 ± 0.3	6.9 ± 0.3	+ 25	0.001-0.005

The data are given ± S.E. and represent 21 experiments with adrenal tissue from control rats and 21 experiments with tissue from rats given ACTH.

sent in the mitochondria during the secondary rate and would be lowered as a consequence of ACTH action. Our initial efforts to determine pregnenolone concentration in the mitochondria and the incubation medium employed Millipore and similar filters for the separation. This approach was unsuccessful due primarily to variable binding of pregnenolone to the filters. Using centrifugal methods it was found that the pregnenolone in the isolated mitochondrial pellet did indeed reach a constant value, but so rapidly that the initial increase could not be detected. These initial time points can be obtained, however, by substracting the values obtained in the mitochondrial supernatant from those in the complete medium. The inability to obtain the initial values in the mitochondria appears to be largely due to some continued pregnenolone synthesis during the isolation of the mitochondrial pellet since in a few experiments in which this was tested the addition of aminoglutethimide an inhibitor of pregnenolone synthesis (15), prior to the centrifugal separation permitted a detection of initial increases. Constant values for mitochondrial pregnenolone were observed throughout the duration of the secondary rate of pregnenolone synthesis. This finding is consistent with the observation that the secondary rate of pregnenolone synthesis in the supernatant fraction is the same as that in the entire medium (Table 2).

It is seen also from the data in Table 2 that more pregnenolone was associated with the mitochondrial pellets ob-

tained from incubations carried out with mitochondria from ACTH treated rats than with mitochondria from control rats. Although 90-95% of the pregnenolone added to an incubation mixture can be recovered in the supernatant after centrifugation, it is still possible that under conditions of high endogenous mitochondrial pregnenolone, some nonspecific binding or retention may occur within the mitochondria. Accordingly, mitochondrial pregnenolone was determined in incubations where corticosterone was the final product and hence, no accumulation of pregnenolone occurred. Under these incubation conditions higher levels of pregnenolone also were found in the mitochondrial pellets from incubations where the mitochondria came from rats given ACTH than from control rats (Table 4). The total pregnenolone in these incubations was found to be too low to be measured by the cycling procedure and the use of radioimmunoassay was required. These low levels which would minimize any nonspecific binding of pregnenolone to the mitochondria could account for the lower values of mitochondrial pregnenolone reported in Tables 4 and 5 compared to those in Table 2.

An additional consideration is that the mitochondrial pregnenolone may be compartmentalized with only that in the membranes, the site of the cholesterol side-chain cleavage enzyme (16), involved in the feed-back inhibition of its synthesis. The determination of pregnenolone in fractions obtained following sonication of the mitochondria showed

TABLE 4

Pregnenolone Levels in the Mitochondrial and Post-Mitochondrial Supernatant Fractions During the Synthesis of Corticosterone

	Experiment 1	Experiment 2
	Pregnenolone pmoles/mg protein	
CONTROL		
Supernatant	2.20	6.13
Mitochondria	0.94	5.51
ACTH		
Supernatant	4.55	13.3
Mitochondria	2.56	10.4

After incubation for 20 min. the contents of the beakers were centrifuged for 5 min. at 9,000 x g and the supernatant and the mitochondrial pellet analyzed. The results are expressed in terms of mitochondrial protein.

TABLE 5 - Pregnenolone levels in sonicated mitochondrial and postmitochondrial supernatant fractions during the synthesis of corticosterone. After incubation for 20 min. aminoglutethimide and cyanoketone were added at a final concentration of 0.43mM and 0.01mM respectively and the contents of the beakers centrifuged at 9,000 x g for 10 min. The supernatant was removed and analyzed and the mitochondrial pellet suspended in sucrose-Tris-EDTA which contained aminoglutethimide and cyanoketone at the above concentrations. This suspension was sonicated in ice with a Branson Sonifier at setting 7, with microtip, for nine 10-s intervals. The sonicate was centrifuged at 9,000 x g for 10 min. to obtain pellet 1 and at 144,000 x g for 60 min. to obtain pellet 2 and the sonicate supernatant. Pellets 1 and 2 were resuspended in sucrose-Tris-EDTA containing aminoglutethimide and cyanoketone.

The values for the original supernatant are expressed in terms of the total mitochondrial protein present and the values for the sonicated mitochondria in terms of the protein present in each fraction.

TABLE 5

Pregnenolone Levels in Sonicated Mitochondrial and Postmitochondrial Supernatant Fractions During the Synthesis of Corticosterone

	Experiment 1	Experiment 2	Experiment 3
	Pregnenolone pmoles/mg protein		
CONTROL			
Supernatant	6.38	9.90	4.14
Sonicated Mitochondria			
Pellet 1	17.2	8.25	3.98
Pellet 2	11.9	10.4	5.43
Supernatant	2.94	4.36	1.85
ACTH			
Supernatant	8.45	18.7	11.4
Sonicated Mitochondria			
Pellet 1	19.2	21.2	17.1
Pellet 2	17.0	32.3	9.58
Supernatant	3.89	14.5	5.31

that ACTH-treatment results in an increase in the pregnenolone content of all the fractions obtained (Table 5).

The rate of corticosterone synthesis was determined also in the presence of various concentrations of 20,22-dihydroxycholesterol to see whether increased amounts of the intermediates of steroidogenic pathway affect the observed biphasic rate curve. A more rapid increase of these intermediates would be expected to result from the metabolism of the dihydroxycholesterol. It is seen in Fig. 4 and Table 6 that in the absence of dihydroxycholesterol the usual biphasic curve is obtained with the primary rate persisting to about 6.5 min. In the presence of two concentrations of dihydroxycholesterol linear increases in the primary rate are seen. These increases, up to about 4.5 min. for the lower concentration and up to about 3 min. for the higher concentration, are proportional to the concentration of dihydroxycholesterol when the rate obtained in the absence of this compound is subtracted as the control value. While it is difficult to determine the exact point at which the primary rate ceases, it is clear that the duration of that rate decreases with increasing dihydroxycholesterol concentration. The change to a slower rate appears to occur in the experiment presented when approximately 12 nmoles of steroid has been formed. In other experiments this change also occurred at a given concentration of synthesized steroid, ranging from 8 to 12 nmoles. Beyond the time when the initial rate slows and up to about

FIG. 4. The effect of dihydroxycholesterol on the kinetics of corticosterone formation. The adrenal mitochondria and microsomes were prepared from rats given ACTH. The dihydroxycholesterol was added in 0.02ml ethanol per 2.5ml of the incubation mixture. In these experiments the reaction was initiated by the addition of Mg^{++} plus succinate after a 10 min. preincubation. Curve A, no dihydroxycholesterol; Curve B, 6.6 nmoles dihydroxycholesterol; Curve C, 13.2 nmoles dihydroxycholesterol.

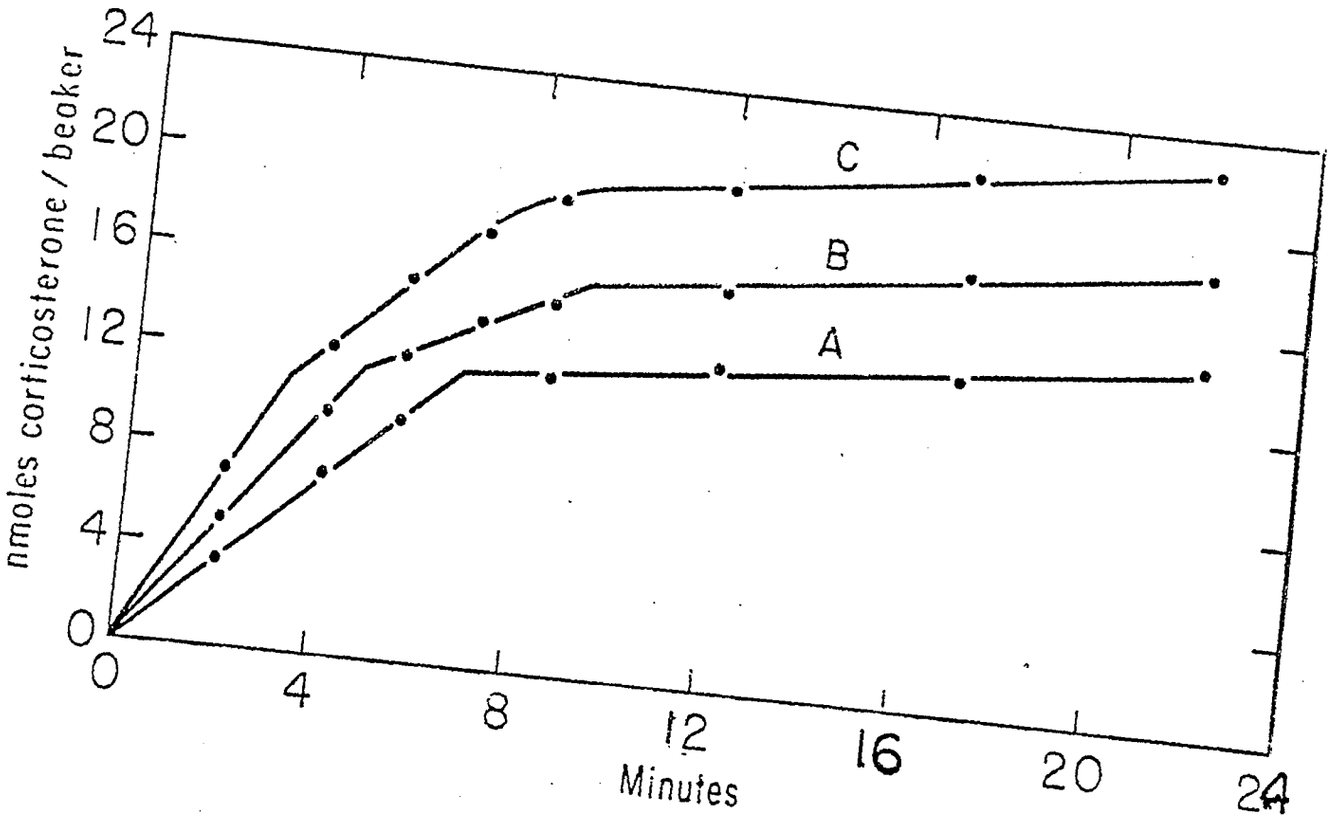
nmoles corticosterone / beaker
0
4
8
12
16
20
24
Minutes
A
B
C

TABLE 6

Numerical data derived from Fig. 4

Curve	Di-OH chol. added	Corticosterone synthesis from di-OH chol.	Total primary rate	Primary rate from di-OH chol.	Intermediate rate	Intermediate rate from di-OH chol.
	nmoles		nmoles corticosterone per min.			
A	0		1.75		0.20*	
B	6.6	7.0	2.53	0.78	1.00	0.80
C	13.2	12.8	3.50	1.75	1.85	1.65

*This value is the secondary rate found in the absence of di-OH chol.

9 min., a new approximate linear rate appears to be formed. Evidence for the existance of these intermediate rates is provided by the fact that when the secondary rate formed in the absence of dihydroxycholesterol is subtracted as the control value, the rates obtained in both curves B and C in Fig. 4 are now proportional to the dihydroxycholesterol concentration and equal to the corresponding initial rates (last column, Table 6). This finding, that the intermediate rates in curves B and C are attributable to the metabolism of the added dihydroxycholesterol, is further supported by the observation that after the completion of the intermediate rate the dihydroxycholesterol has now been exhausted (about 100% conversion to corticosterone) and the rate of corticosterone synthesis is that found in the absence of added dihydroxycholesterol. A 10 min. preincubation with dihydroxycholesterol was required to obtain linear, initial, kinetics.

DISCUSSION

The rate of pregnenolone synthesis characteristically shows a biphasic curve with a rapid primary rate and a slower secondary rate. ACTH administration, in vivo, affects this curve by increasing both primary and secondary rates but the percentage increase is greater for the secondary rate. In addition, the effect of ACTH is manifest in an increase in the duration of the primary rate (Tables 2 and 3).

These characteristics can be explained by several models for ACTH action. One model is based on the observations that pregnenolone can act as an inhibitor of its own synthesis from cholesterol but not from 20α-hydroxycholesterol (17-19) and that substances which cause mitochondria to swell stimulate pregnenolone synthesis (20). It has been suggested that ACTH affects the mitochondrial membranes so as to increase the rate of efflux of pregnenolone (7). In this view the primary rate would reflect a less inhibited state resulting from an initially low level of intramitochondrial pregnenolone. These levels of pregnenolone would increase with time so that the primary rate becomes inhibited and the secondary rate initiated. An ACTH-induced increase in the rate of mitochondrial pregnenolone efflux would lower the pregnenolone concentration and hence release the inhibition, and in addition, result in an increase in the time required to establish a new inhibitory level of mitochondrial pregnenolone. However, a critical prediction of this model, namely a decrease in mitochondrial pregnenolone following ACTH stimulation of steroidogenesis, has been found not to hold under a variety of experimental conditions (Tables 2,4,5). Other investigators have also found an increase in mitochondrial pregnenolone after ACTH stimulation under different experimental conditions (21,22). These data make unlikely an action of ACTH on the rate of efflux of mitochondrial pregnenolone, unless one postulates a highly

localized effect, a not very profitable consideration at present.

Another model which proposes that the limiting and hence ACTH sensitive step, in pregnenolone synthesis is the rate at which mitochondrial cholesterol is transported to, or binds to, the cholesterol side-chain cleavage enzyme can also explain much of these data (8,9,23,24). The primary rate would then reflect the cholesterol readily available to, or already bound to, the enzyme and the slower secondary rate, its transport or binding to the enzyme. The increase in the duration of the primary rate upon ACTH stimulation would be the result of an increased amount of cholesterol in the enzyme compartment. This model, however, presents no ready explanation for the decrease in the duration of the primary rate found in the presence of dihydroxycholesterol.

The possible role of an inhibitor in the regulation of steroidogenesis is indicated by the experiment presented in Fig. 4 and Table 6 . It is seen that in the presence of increasing concentrations of dihydroxycholesterol there is a progressive decrease in the duration of the primary slope with no change in its magnitude and hence a decrease in the time needed to establish the slower secondary slope, again with no change in its magnitude. These, and the other data are consistent with a model in which the transition from the primary rate to the slower secondary rate is a reflection of the accumulation of an inhibitory substance. Its

more rapid accumulation due to the metabolism of dihydroxycholesterol would result in the shortening of the inflection point time. The regulation of the inhibition need not be a function of the concentration of the inhibitor but rather the susceptibility of the side-chain cleavage enzyme to the inhibitor. In this view the primary rate would reflect the uninhibited rate, the secondary rate, the inhibited rate and the inflection point time would be the time needed to build up the concentration of the inhibitor required for maximal inhibition of the side-chain cleavage enzyme under a given set of conditions. The action of ACTH would then be to modify the structure or conformation of the cholesterol side-chain cleavage enzyme so that there is a decreased susceptibility of the enzyme to the inhibitor.

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SECTION II - THE INHIBITION OF RAT ADRENAL STEROIDOGENESIS BY ADENINE CONTAINING COMPOUNDS

INTRODUCTION

The synthesis of corticosterone from cholesterol in the rat adrenal cortex consists of a series of reactions which occur in the mitochondria and the microsomes. The transformation of endogenous cholesterol to pregnenolone takes place in the mitochondria (1); the formation of progesterone from pregnenolone and the 21-hydroxylation of progesterone to yield deoxycorticosterone occur in the microsomes (2,3); and the final 11β-hydroxylation of deoxycorticosterone to give corticosterone takes place in the mitochondria (2). There is thus present an interaction between two organelles to accomplish the synthesis of the final product. There is evidence that there are regulatory factors involved in this complex series of reactions in addition to the primary regulation by ACTH at the transformation of cholesterol to pregnenolone (4,5). Thus, it has been found that 11β-hydroxylation of deoxycorticosterone inhibits the conversion of cholesterol to pregnenolone, but the reverse situation does not occur (6). Pregnenolone can inhibit its own formation from cholesterol (7). Ca^{++} stimulates pregnenolone synthesis (8) and 11β-hydroxylation of deoxycorticosterone (9). Reduced NAD^{+} inhibits the formation of progesterone from pregnenolone and this inhibition can be reversed by ascorbic acid among other substances (10). Both NAD^{+} and NADH stimulate the Δ5-3-keto steroid isomerase (11). It is not known what physiological func-

tions these modifications of the steroidogenic enzyme reactions may have, but the existence of such a number suggests the possibility that this sequence of reactions may well be functionally a highly integrated system. In addition, a number of investigations have been carried out on the relationship between steroid 11β-hydroxylation and oxidative phosphorylation (12-16).

In this section an inhibition by ADP (ATP) of corticosterone synthesis from endogenous precursors is described. Several of the properties of this inhibition have been investigated and it has been found to take place only in the 11β-hydroxylation of deoxycorticosterone.

MATERIALS AND METHODS

Rat adrenal glands were removed, homogenized and the homogenate fractionated according to the procedure described previously (17) except that the homogenization medium consisted of 0.25M sucrose which contained 1mM EDTA and 12mM Tris buffer at pH7.5. The preparation of mitochondria contained, on the average, about 2.0-2.5mg protein per ml and the microsomes about 1.8-2.1mg protein per ml.

The incubation conditions in cell-free systems for the synthesis of corticosterone from endogenous precursors (mitochondria plus microsome) and from deoxycorticosterone (mitochondria), the synthesis of deoxycorticosterone from progesterone (microsomes), the formation of progesterone from pregnenolone (microsomes) and the synthesis of preg-

nenolone from endogenous precursors (mitochondria) have been given elsewhere (17). All incubations were carried out at 20°C in the presence of phosphate and succinate unless otherwise indicated. The analytical methods used have been described (17) except that pregnenolone was determined by a different procedure (18). In most experiments the tissue was obtained from rats treated with ACTH (17) and unless otherwise indicated in the data corticosterone synthesis from endogenous precursor is presented.

RESULTS

The effects of ADP and ATP on the time-course of synthesis of corticosterone from endogenous precursor in a cell-free system are shown in Fig. 5. The usual biphasic rate curve is obtained in the absence of ADP or ATP (17). In the presence of these adenine nucleotides an initial inhibition of corticosterone synthesis is observed, followed by a recovery from this inhibition so that essentially no effect is found after 20 min. of incubation. The extent and duration of the inhibition depends on the nucleotide and its concentration, ADP being more effective than ATP.

The inhibitory effects of various nucleosides and nucleotides are given in Table 7. It is seen that in the adenine series ADP is most effective followed by ATP and that AMP and adenosine are about equally effective. The nucleosides and nucleotides of guanine, uracil and cytosine are approximately equally effective with no inhibition by the nucleosides and with considerably less inhibition than ADP by the various nucleotides. In all cases the inhibitory effects decrease with an increase in time of incubation. Since ADP and ATP are metabolically readily interconvertible in this incubation system, they have been used interchangeably in many of the following experiments.

A number of characteristics of the inhibition of corticosterone synthesis by ADP (ATP) have been determined. It is seen from the data in Table 8 that while phosphate has only

FIG. 5. The time-course of inhibition of corticosterone synthesis by ADP and ATP. Curve 1, no ADP or ATP present; curve 2, 0.1mM ATP; curve 3, 0.1mM ADP; curve 4, 1.0mM ATP; curve 5, 1.0mM ADP.

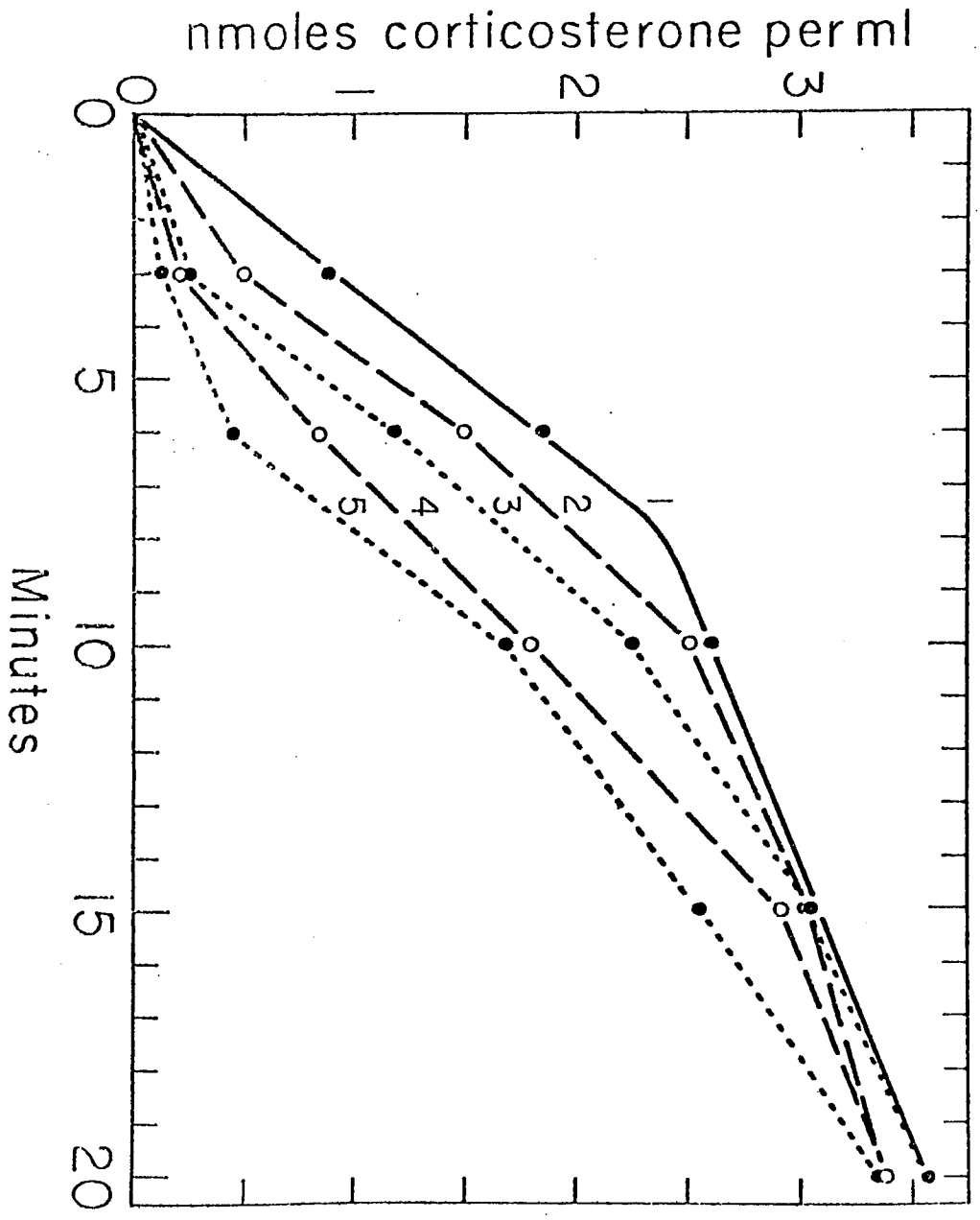
nmoles corticosterone per ml
0
1
2
3
Minutes
0
5
10
15
20
1
2
3
4
5

TABLE 7 - The inhibition of corticosterone synthesis by various/nucleosides and nucleotides. When derivatives of bases other than adenine were tested, ADP was included in the experiment to provide a standard for the inhibitions caused by the other substances. The values, in nmoles of corticosterone per ml, for incubations with no nucleosides or nucleotides present for 3 min. and 9 min. incubation times are for experiment 1, 1.02 and 2.67; experiment 2, 0.87 and 2.05; experiment 3, 1.09 and 2.89; experiment 4, 1.11 and 2.77.

TABLE 7

The Inhibition of Corticosterone Synthesis by Various Nucleosides and Nucleotides

EXPERIMENT		1		2		3		4	
Addition		Adenine		Guanine		Uracil		Cytosine	
Incubation time		3 min	9 min	3 min	9 min	3 min	9 min	3 min	9 min
	mM	Percent inhibition of corticosterone synthesis							
Nucleoside	0.1	22	5	0	0	0	0	0	0
"	2.0	78	13	0	0	0	0	0	0
Nucleoside-3'-P	0.1	0	0						
"	2.0	11	0						
Nucleoside-5'-P	0.1	28	5	0	0	0	0	0	0
"	2.0	72	15	15	0	12	8	13	10
Nucleoside-5'-diP	0.1	84	24	21	9	12	10	25	10
"	2.0	100	44	26	9	29	11	25	13
Nucleoside-5'-triP	0.1	66	13	15	0	12	8	6	3
"	2.0	100	34	21	6	24	8	25	15
ADP	0.1			64	15	53	13	69	15
"	2.0			91	29	86	31	92	38

TABLE 8

The Effect of Phosphate on the Inhibition by ADP and ATP

Incubation time	3 minutes		6 minutes	
ADDITIONS	Corticosterone nmoles/ml	Inhibition %	Corticosterone nmoles/ml	Inhibition %
No phosphate				
-	0.96		1.75	
ADP, 0.1mM	0.90	6	1.75	0
ADP, 1.0mM	0.72	25	1.69	0
ATP, 0.1mM	0.90	6	1.80	0
ATP, 1.0mM	0.81	16	1.81	0
1.7mM phosphate				
-	0.96		1.98	
ADP, 0.1mM	0.54	44	1.63	18
ADP, 1.0mM	0.30	69	1.33	33
ATP, 0.1mM	0.60	38	1.63	18
ATP, 1.0mM	0.42	56	1.44	27
12mM phosphate				
-	1.02		2.04	
ADP, 0.1mM	0.12	88	1.27	38
ADP, 1.0mM	0.12	88	0.54	74
ATP, 0.1mM	0.36	65	1.51	26
ATP, 1.0mM	0.12	88	0.75	63

a slight stimulatory effect on corticosterone synthesis from endogenous precursors in a cell-free system, its presence is required for the inhibition to take place. The decrease in the inhibition with an increase in incubation time occurs at all phosphate concentrations. It was found that a decrease in succinate concentration from the 12mM routinely used to 5mM, results only in a slight decrease (10%) in corticosterone synthesis but an increase in the inhibition by ATP from 40% to 62% after 3 min. of incubation. The inhibition is prevented by the presence of 0.5mM potassium atractyloside. The inhibition is also eliminated if the ATP is added to the reaction mixture 3 min. after the start of the incubation (Table 9). The use of adrenal mitochondria and microsomes from rats given ACTH in vivo 15 min. before killing has little effect on the inhibition, although there is a suggestion that the ACTH administration results in a somewhat greater and more protracted inhibition.

Several of these observations, particularly the elimination of the inhibition by atractyloside and the increase in the inhibition brought about by a decrease in succinate concentration suggest that a reaction located within the mitochondria is involved. A survey of the reactions of the major steps in the synthesis of corticosterone, i.e., the conversion of cholesterol to pregnenolone, the formation of progesterone from pregnenolone, the 21-hydroxylation of progesterone to give deoxycorticosterone and finally,

TABLE 9

The Effect of the Late Addition of ATP

on Corticosterone Synthesis

ATP	Time after start of incubation of ATP addition	INCUBATION TIME			
		3 min.	6 min.	9 min.	12 min.
		nmoles corticosterone/ml			
0		0.79	1.76	2.82	3.00
2mM	0 min.	0.18	0.57	1.41	2.20
2mM	3 min.		1.76	2.73	3.08
2mM	6 min.			2.82	3.08

the 11β-hydroxylation of deoxycorticosterone to yield corticosterone, showed that ATP inhibits only the conversion of deoxycorticosterone to corticosterone (Tables 10 and 11). The other mitochondrial reaction, the synthesis of pregnenolone, was not affected. It is also seen in Table 11 that the extent of 11β-hydroxylation decreases with increasing deoxycorticosterone concentration in confirmation of the observations made by Hayano and Dorfman (19). The percentage of inhibition by ATP also decreases with increasing deoxycorticosterone concentrations so that at 100μM virtually no inhibition is seen. It is to be noted, however, that the absolute amounts of corticosterone formed in the presence of ATP remains essentially constant at the shorter incubation times throughout the range of deoxycorticosterone concentrations.

Several additional lines of evidence also indicate that the inhibition by ADP (ATP) of corticosterone synthesis from endogenous precursors can be accounted for by the inhibition of 11β-hydroxylation. In both systems the inhibition decreases with time of incubation (Fig. 5 and Table 11). As is seen from the data in Table 12 , the presence of glucose plus hexokinase increases the inhibition caused by ATP in both systems, while the addition of PEP plus pyruvate kinase not only erases the inhibition but in the case of 11β-hydroxylation results in a stimulation. The omission of phosphate also eliminates the ATP inhibition of 11β-hydroxylation, as was found for corticosterone syn-

TABLE 10

The Effect of ATP on Enzyme Systems of Steroidogenesis

ASSAY		ATP
	nmoles product/min/mg protein	
Pregnenolone Synthesis	0.33	0.33
Pregnenolone to progesterone	5.13	4.78
Progesterone to Deoxycorticosterone	1.85	1.81

ATP when present was 2mM.

TABLE 11

The Effect of ATP on the Conversion of 11-deoxycorticosterone to corticosterone

Deoxycorticosterone µM	ATP	Incubation time 3 min.	6 min.	10 min.
		nmoles corticosterone/ml		
12.5	-	1.98	5.92	10.0
	+	0.76	3.36	9.43
	Inhibition	62%	43%	6%
25	-	1.52	4.72	10.0
	+	0.76	3.34	8.85
	Inhibition	50%	29%	11%
50	-	1.22	3.34	7.18
	+	0.76	2.74	6.53
	Inhibition	38%	18%	9%
100	-	0.91	3.04	6.53
	+	0.84	2.90	6.53
	Inhibition	8%	0	0

ATP when present was 2mM.

TABLE 12 - The effect of ATP dephosphorylation, ADP phosphorylation and phosphate on the ATP inhibition of corticosterone synthesis. When present, ATP was 2mM; glucose 12mM; PEP (phosphoenolpyruvate) 5mM; crystalline hexokinase, 12 units per ml incubation medium; and crystalline pyruvate kinase, 5 units per ml incubation medium. Incubation time was 3 min. Corticosterone synthesis was determined in condition A from endogenous precursors in the presence of mitochondria and microsomes, and in condition B from added 11-deoxycorticosterone in the presence of mitochondria.

TABLE 12

The Effect of ATP Dephosphorylation, ADP Phosphorylation and Phosphate on the ATP Inhibition of Corticosterone Synthesis

ADDITIONS	A		B	
	Corticosterone nmoles/ml	Change %	Corticosterone nmoles/ml	Change %
-	1.64		2.90	
ATP	0.66	-60	1.92	-34
Glucose + hexokinase	1.58		2.42	
Glucose + hexokinase + ATP	0.40	-70	1.12	-54
PEP + pyruvate kinase	1.25		4.18	
PEP + pyruvate kinase + ATP	1.18	0	6.12	+46
P_i omitted			1.77	
P_i omitted + ATP			2.10	+18

thesis from endogenous precursors (Table 8). Since the omission of phosphate reduced the 11β-hydroxylation of added deoxycorticosterone, the stimulation of this reaction by ATP in the absence of phosphate may be a consequence of phosphate derived from the ATP. A stimulation of 11β-hydroxylation by phosphate has been reported by others (15). The addition of oligomycin, which would be expected to impair the rate of mitochondrial phosphorylation of ADP, results only in a partial reversal of the inhibition (Table 13). Higher concentrations of oligomycin inhibited corticosterone synthesis.

The above experiments were carried out with succinate as the source of electrons for the formation of NADPH required for steroid hydroxylation. This involves energy dependent processes such as reverse electron transport and transhydrogenation. Steroid hydroxylation can be supported by isocitrate by an energy independent process (15) presumably by the NADP-linked isocitrate dehydrogenase. The effect of ADP on isocitrate supported corticosterone synthesis is given in Table 14. It is seen that little or no inhibition is observed. In the same experiment with succinate replacing isocitrate ADP caused a 67% inhibition in corticosterone synthesis from endogenous precursors (condition A) and a 42% inhibition in corticosterone synthesis from added corticosterone (condition B). Succinate was about 75% as effective as isocitrate in supporting corticosterone synthesis.

TABLE 13

The Effect of Oligomycin on the Inhibition of Corticosterone Synthesis by ADP

ADDITIONS	A		B	
	Corticosterone nmoles/ml	Change %	Corticosterone nmoles/ml	Change %
-	1.08		1.80	
Oligomycin	1.08	0	1.80	0
ADP	0.42	-61	0.71	-61
ADP + oligomycin	0.77	-29	1.16	-36

Conditions A and B are described in the legend to Table 12. Oligomycin was present at a concentration of 0.84µg per ml incubation medium A and 1.6µg in B. The concentration of ADP was 2mM. The incubation time was 3 min.

TABLE 14

The Effect of ADP on Isocitrate Supported

Corticosterone Synthesis

ADDITIONS	A Corticosterone nmoles/ml	A Change %	B Corticosterone nmoles/ml	B Change %
-	1.35		3.00	
ADP	1.13	-16	3.03	0
Antimycin A	1.28		2.89	
ADP + Antimycin A	1.13	-12	2.84	0

DL-Isocitrate was present at 24mM, ADP at 2mM, and antimycin A at 4.2µg per ml incubation medium. The incubation time was 3 min. Conditions A and B are described in the legend to Table 12. In concomitant incubations with succinate in place of the isocitrate, ADP caused a 67% inhibition in A and a 42% inhibition in B.

DISCUSSION

The succinate supported synthesis of corticosterone from endogenous precursors in a rat adrenal cell-free system consisting of mitochondria, microsomes and appropriate cofactors has been found to be inhibited by the addition of ADP or ATP. The evidence presented indicates that this inhibition takes place at the mitochondrial 11β-hydroxylation of deoxycorticosterone to form corticosterone. Of the four major steps in rat adrenal steroidogenesis only this reaction and not the formation of pregnenolone, progesterone, or deoxycorticosterone is inhibited. In addition, many of the characteristics of the inhibition of the transformation of deoxycorticosterone to corticosterone are similar to those found in the formation of corticosterone from the endogenous precursors. Thus, in both, the inhibition decreases with time of incubation, oligomycin causes a partial reversal of the inhibition, the presence of glucose plus hexokinase increases the inhibition while the presence of phosphoenolpyruvate plus pyruvate kinase reverses the inhibition, and the presence of phosphate is required for the inhibition to take place. In addition, in both cases the inhibition is greatly reduced or eliminated if succinate is replaced by isocitrate. These data support the conclusion that in the succinate supported steroidogenic sequence of reactions only 11β-hydroxylation is inhibited by ADP or ATP.

A possible explanation for an inhibition by ADP(ATP) of a mitochondrial hydroxylation reaction dependent upon the oxidation of a Krebs cycle acid would be a competition for reducing equivalents by the hydroxylating system and oxidative phosphorylation. A number of the observations can support this interpretation. Thus, the inhibition requires phosphate, is partially reversed by oligomycin and is increased when the succinate concentration is decreased. Furthermore, maintenance of high ADP levels by the addition of glucose plus hexokinase increases the inhibition, and in the reverse situation where ADP concentration is reduced, by the addition of phosphoenolpyruvate plus pyruvate kinase, the inhibition is eliminated. Also, in support of this interpretation is the essential elimination of the inhibition when succinate is replaced by isocitrate. In this case the generation of reducing equivalents required for steroid hydroxylation does not involve energy generating systems (15). These observations would indicate that the inhibitor is ADP rather than ATP, a suggestion in keeping with the finding that, in general, ADP is more inhibitory than ATP. The inability of other investigators (14,15) to find an inhibition of 11β-hydroxylation by ADP may be due to the high concentrations of deoxycorticosterone used or to the absence of phosphate.

However, an explanation of the inhibition solely on the basis of a competition for reducing equivalents does not appear to account for the reversibility of the inhibition

with increasing time of incubation and for the absence of the inhibition if ATP is added shortly after the start of the incubation. These observations suggest an effect on steroidogenesis by a mechanism in addition to the postulated competition. In addition, experiments presented in section III and IV have shown that ADP will inhibit NADPH supported 11β-hydroxylation in disrupted mitochondria. The finding that the ADP (ATP) inhibition of 11β-hydroxylase decreases with increasing incubation times suggests the possible accumulation of a substrate which either modifies or counteracts that inhibition. The extent of the accumulation of such a substance under the more dynamic conditions present during in vivo hydroxylation can now determine the magnitude of the ADP inhibition. This inhibition might then impose a regulatory process on steroidogenesis secondary to the ACTH sensitive step, the conversion of cholesterol to pregnenolone in the mitochondria. It is of interest that this latter reaction is not inhibited by ADP when supported by succinate. This would suggest that this reaction can more effectively compete with the cytochrome chain for reducing equivalents than can 11β-hydroxylation.

The data do not permit a single explanation for the characteristics of the inhibition produced by ADP (ATP). Both a competition for reducing equivalents by the hydroxylating system and oxidative phosphorylation, and a direct effect of the adenine nucleotides on the hydroxylase system must be considered.

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SECTION III - THE INHIBITION BY ADP OF NADPH-SUPPORTED ADRENAL STEROID 11β-HYDROXYLATION

INTRODUCTION

Accumulating evidence suggests the possibility that, in the adrenal cortex, the sequence of reactions from cholesterol to corticosterone or cortisol may be functionally a highly integrated system. In addition to the primary control by the adrenocorticotropic hormone (ACTH) which takes place at the transformation of cholesterol to pregnenolone, a series of secondary controls of the steroidogenic pathway appear to be present. Thus, a number of investigations have shown a relationship between steroid 11β-hydroxylation and oxidative phosphorylation (1-5) and steroid 11β-hydroxylation can inhibit the conversion of cholesterol to pregnenolone, but the reverse situation does not occur (6). Ca^{++} has been found to stimulate both pregnenolone synthesis (7) and 11β-hydroxylase (8). Reduced NAD^{+} inhibits the formation of progesterone from pregnenolone and this inhibition can be reversed by ascorbic acid and other substances (9). Both NAD^{+} and NADH stimulate Δ5-3-keto-steroid isomerase (10).

In the previous section it was found that 11β-hydroxylation of deoxycorticosterone (DOC) to form corticosterone in rat adrenal mitochondria is inhibited by ADP. Much of the data can be explained on the basis of a competition for reducing equivalents between the hydroxylase system and the cytochrome chain. However, some observations suggested the possibility that ADP may also affect the hydroxy-

lase system directly. In this section data is presented which indicate that ADP can indeed directly inhibit steroid 11β-hydroxylation.

MATERIALS AND METHODS

Mitochondria were prepared from rat adrenals as described previously (11). Following the second 0.25M sucrose wash, the mitochondrial pellet was suspended in a volume of 12mM Tris, pH7.5, equal to that of the original homogenate (90mg wet weight of tissue per ml) and contained 2.0 to 2.5mg of protein per ml as determined by the method of Lowry et al. (12).

The complete system for the 11β-hydroxylation of deoxycorticosterone (DOC) to form corticosterone consisted of 0.2ml of the mitochondrial preparation, 36mM Tris, pH7.5, 20mM $CaCl_2$, 30μM DOC, 0.07ml of NADPH prepared by a preliminary incubation for 15 min. at room temperature of a solution containing, per ml, 25μmoles of $NaNADP^+$, 70 of μmoles Na_2 glucose-6-P and about 2,000 units of glucose-6-P dehydrogenase, other additions as indicated and 0.25 M sucrose to a final volume of 1.25ml. The DOC was added in 0.01ml ethanol and an incubation control with 0.01ml ethanol without DOC was routinely carried out. The incubations were carried out at 20°C in a Dubnoff shaking incubator for 6 min., at which time 0.4ml aliquots were delivered into 3ml of dichloromethane, and after shaking and centrifugation, 2ml of the dichloromethane taken for

corticosterone determination by a modification (11) of the fluorometric method of Glick et al. (13).

RESULTS

The incubation conditions used permit the 11β-hydroxylation of deoxycorticosterone to be supported directly by exogenous NADPH. This would obviate a competition by the hydroxylase and the cytochrome chain for reducing equivalents derived from a Krebs cycle acid such as succinate. It is seen from the data in Table 15 that 11β-hydroxylase supported by NADPH as the source of electrons is inhibited by ADP and ATP. ADP is more inhibitory than ATP, as was found with intact mitochondria and that a clear inhibition is seen at concentrations as low as 50μM.

Ca^{++}, which is present in these incubations, is known to induce swelling in adrenal mitochondria (8,14) and ADP and ATP have been found to inhibit this swelling (14). The inhibition of the 11β-hydroxylase by ADP may result from a modification of the mitochondrial membranes so that the entry of NADPH is affected. Several experiments were carried out to explore this possibility. The data in Table 16 show that the presence of several different types of detergents, which would be expected to disrupt mitochondrial structure, the inhibition by ADP persists. In the presence of some detergents the sensitivity of the hydroxylase to Ca^{++} changes and the Ca^{++} concentration has been adjusted to obtain maximal activity.

TABLE 15

The Effect of ADP and ATP on the Formation of Corticosterone from Deoxycorticosterone

Nucleoside concentration mM	ADP		ATP	
	nmoles corticosterone per ml	% change	nmoles corticosterone per ml	% change
0	13.0		13.0	
0.01	13.0	0	13.4	0
0.05	9.07	-30	12.1	-7
0.10	6.34	-51	11.5	-12
0.50	2.88	-78	7.06	-46
1.0	1.58	-88	4.90	-62

TABLE 16

The Inhibition of 11β-Hydroxylation by ADP in the Presence of Various Detergents

ADDITION	nmole corticosterone/ml	ADP	% change
Lubrol	18.0	1.8	-90
Triton X-100	13.8	1.5	-89
Deoxycholate	18.1	5.8	-68

ADP, when present, was 2mM. The concentrations of the detergents, per ml of incubation medium, were Lubrol, 40µg; Triton X-100, 80µg; Na deoxycholate, 80µM. Ca^{++} concentrations in incubations with Lubrol was 20mM, and in those with Triton X-100 and deoxycholate were 8mM.

In the experiments presented in Table 17 the effects on the ADP inhibition of preincubation of the mitochondria under various conditions has been determined. It is seen that the ADP inhibition is still present after a 10 min. preincubation in the presence of Ca^{++}, NADPH and ADP or in the presence of Ca^{++} and NADPH. There is a small decrease in the inhibition under the latter preincubation condition.

DISCUSSION

A number of observations indicate that the ADP effect is due to an action directly upon the hydroxylase system. The ADP inhibition persists in the presence of various detergents as well as when the mitochondria are preincubated with Ca^{++} and NADPH where the possibility of an ADP induced impairment of NADPH entry into the mitochondrial membranes is not present. In support of this conclusion is the observation that while ADP is more inhibitory than ATP, ATP is more effective than ADP in preventing the Ca^{++} induced swelling of adrenal mitochondria (14). The ADP inhibition of 11β-hydroxylase activity is somewhat less when the mitochondria are preincubated with Ca^{++} and NADPH (77%) than when ADP is also present during this preincubation (89%). This may indicate a possible minor effect of ADP upon NADPH entry into the mitochondrial membranes. Further work will be required to clarify the mechanism of the ADP inhibition as well as the Ca^{++} stimulation of this enzyme system.

TABLE 17

The Effect of Preincubation in the Absence and Presence of ADP on the Inhibition of 11β-Hydroxylase by ADP

Preincubation	—	ADP	% change
	nmoles corticosterone/ml		
A	17.2	1.5	-91
B	22.2	5.2	-77
C	21.3	2.3	-89

Preincubation: A, none. B, 10 min. with the complete system except for DOC. ADP, when present, was added in the final incubation. The reaction was started with DOC. C, the preincubation was the same as in B except that ADP, when added, was present during the preincubation. The reaction was started with DOC.

ADP, when present, was 2mM.

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SECTION IV - Ca^{++} STIMULATION AND ADP INHIBITION OF 11β-HYDROXYLATION OF DOC IN A BEEF ADRENAL CORTICAL MITOCHONDRIAL PREPARATION

INTRODUCTION

The conversion of cholesterol into corticosteroids by the adrenal cortex involves enzymatic activities from both the mitochondria and the endoplasmic reticulum. The mitochondrial contribution includes the activities of both the initial step, the side-chain cleavage of cholesterol to form pregnenolone (1), and the last hydroxylation in which DOC is converted to corticosterone (2). Steroid C-21 (3) and C-17 (4) hydroxylation and the hydroxysteroid dehydrogenase/isomerase (5,6) activities are believed to be associated with the endoplasmic reticulum. ACTH regulates the overall rate of steroidogenesis by stimulating the rate of the side-chain cleavage of cholesterol in the mitochondria (7,8). A relationship between the side-chain cleavage of cholesterol and the 11β-hydroxylation of DOC in the mitochondria is evident from the finding that pregnenolone synthesis was inhibited in the presence of DOC (9), an inhibition which involves a competition for reducing equivalents as well as other unknown elements. Therefore, it appears that any regulation of the 11β-hydroxylase reaction may well affect pregnenolone synthesis and be involved, as a secondary control, in the overall regulation of steroidogenesis.

One such possible regulator is Ca^{++} which has been shown by Pèron *et al*. (10) to stimulate NADPH-supported 11β-hydroxylation in rat adrenal mitochondria. This stimulation was found to be closely related to mitochondrial

swelling and hence, it was suggested that it functioned by making NADPH or DOC available to the enzyme system. However, they indicated that factors other than the swelling effect are involved also in this Ca^{++} stimulation. Another possible regulator of 11β-hydroxylase system is ADP. The data presented in Section III showed that ADP inhibited the Ca^{++} stimulated 11β-hydroxylase activity under conditions in which the involvement of NADPH entry into the mitochondria was eliminated.

In this Section the effects of both Ca^{++} and ADP on the 11β-hydroxylation of DOC in beef adrenal cortical mitochondrial preparation stored in 50% glycerol were investigated. Both effects were found to be localized at the reduction of cytochrome P450 by adrenodoxin.

MATERIALS AND METHODS

Preparation of mitochondria: Beef adrenal were obtained in ice from a slaughter house. The adhering fat was removed and the medulla was dissected from the cortex. The cortex was then scraped off into a beaker containing 0.25M sucrose, 1.0mM EDTA and 12mM Tris buffer pH7.5. The cortex suspension was homogenized with 10 volumes of the above sucrose medium in a glass homogenizer fitted with a Teflon pestle for a total of 4-7 passes. The homogenate was centrifuged at 755 x g for 10 min. and the pellet was discarded. The mitochondria were then sedimented from the supernatant by centrifugation at 7,710 x g for 10 min. The mitochondrial pellet obtained was resuspended in the homo-

genization medium to the original volume and centrifuged at 5,900 x g for 10 min. The resulting pellet was washed by the same procedure for two more times. After the third wash the mitochondrial pellet was suspended in 12mM Tris buffer pH7.5 to half of the original homogenization volume and an equal volume of glycerol was then added to this mitochondrial suspension. The mitochondrial preparation which contained 1.9-2.3mg protein/ml as determined by the method of Lowry et al. (11) was stored at -20°C. The presence of glycerol was found to interfere with some of the assay procedures employed in this study and the glycerol was removed from the mitochondrial preparations used in such assays. Three to four volumes of 12mM Tris buffer pH7.5 were added to one volume of the stored 50% glycerol mitochondrial preparation and after mixing the preparation was centrifuged at 48,200 x g for 10 min. The supernatant was discarded and the mitochondrial pellet was resuspended to the same volume in 12mM Tris buffer, pH7.5. The mitochondrial suspension was centrifuged again at 48,000 x g for 10 min. and the pellet was suspended in the Tris buffer to the desired protein concentration. The glycerol removal resulted in a 40-50% loss of the mitochondrial protein with no, or little, change in the total 11β-hydroxylase activity. The effects of both Ca^{++} and ADP on corticosterone synthesis in the glycerol free mitochondria were maintained, but lower concentrations of Ca^{++} and ADP were found to be needed, for maximal effects, than when the glycerol preparations were used.

Incubation conditions for corticosterone synthesis: Incubation were carried out in beakers at 20°C and the incubation medium consisted of 37.5mM Tris buffer pH7.5, 45nmoles DOC in 10µl ethanol and 0.07ml of an NADPH generating system, prepared by a preliminary incubation for 15 min. at room temperature of 71µmoles of Na-glucose-6-phosphate, 24µmoles NaNADP, both at pH7.5, and about 2,000 units of yeast glucose-6-phosphate dehydrogenase per ml. Other addition are as indicated in the text and the final incubation volume of 1.2ml was made up by the addition of water. Aliquots, taken at different incubation times, were delivered into dichloromethane and corticosterone analysis was carried out as previously described, (12).

^{14}C-Deoxycorticosterone binding: The binding experiments were carried out essentially according to the procedure of Stare and Vignais (13). One-half ml of the glycerol free mitochondrial preparation (1.55mg protein in one experiment and 0.85mg protein in the second experiment) in a total volume of 2.0ml containing variable concentrations of ^{14}C DOC (from 5×10^{-8} to 1×10^{-5}M) were incubated in a centrifuge tube at 0°C. Ca^{++} and ADP, when present, were at a final concentration of 5.0mM and 3.0mM respectively. After 10 min. at 0°C the free and the bound steroid was separated by centrifugation at 48,200 x g for 10 min. One ml aliquots of the supernatant were taken out for counting and the pellet were dissolved in one ml of 2% S.D.S. of which 0.8ml aliquots were taken for counting. Standards

were run by adding known counts of ^{14}C DOC to both the supernatant and the dissolved pellet obtained from incubations which contained no DOC before the centrifugation. The scintillation fluid used was that of Hochberg et al. (14). The bound DOC (nmoles/mg protein) was plotted against the ratios of the bound/free DOC and the binding curve obtained was resolved to the specifically and non-specifically bound components by the method of Stare and Vignais (3).

Difference spectroscopy: Difference spectra were recorded with a Cary 15 spectrophotometer. Equal volumes of the glycerol free mitochondrial preparation (2.6mg protein) were used in the sample and reference cuvettes of 1.0 cm path length. DOC was added in ethanol to sample cuvettes and an equal amount of ethanol (10µl) was added to reference cuvettes. The total volume in the cuvettes was brought up to 3.0ml by the addition of 12mM Tris solution pH7.5. Ca^{++} and ADP, when present, were at the final concentrations indicated in Table 23.

Enzymatic assays of the components of cytochrome P450 electron transporting chain: NADPH diaphorase was assayed according to the procedure of Masters et al.(15) by measuring the decrease in absorbance of dichlorophenolindophenol (DCIP) at 600nm. The reaction mixture consisted of 50nmoles of DCIP in 0.05ml, 0.5µmoles KCN in 0.05ml, 0.18mg protein of glycerol free mitochondrial preparation in 0.025ml and 0.5ml of the indicated concentrations of Ca^{++} and/or ADP. The final volume in both reference and sample cuvettes was

made up to 1.1ml by adding 12mM Tris buffer, pH7.5. The reaction was started by adding 100nmoles of NADPH, in 5µl, to the sample cuvette. The reaction rate was recorded with a Cary 15 spectrophotometer at 600nm. NADPH-cytochrome c reductase activity was assayed according to the method of Masters et al. (15) by using the above reaction mixture, except that 40nmoles of cytochrome c were present instead of DCIP. The reaction was also started by adding 100nmoles of NADPH, in 5µl, to the sample cuvette and was recorded at 550nm with a Cary 15 spectrophotometer.

NADPH-cytochrome P450 reductase activity was measured by the method of Gigon et al. (16) by monitoring the formation of cytochrome P450-CO at 450 and 430nm in a Perkin Elmer dual wave length spectrophotometer. The reaction mixture consisted of 1.5mg of glycerol free mitochondrial protein, 12mM Tris, pH7.5, and other additions as indicated in the text to a final volume of 3.0ml. After gassing 11 min. with CO, the reaction was initiated by adding 100µM NADPH to the sealed cuvette. NADPH was added in 5µl. The rate of cytochrome P450 reduction was calculated using the initial linear part of the reduction curve and a millimolar extinction coefficient of $91mM^{-1}$ cm^{-1} [Omura and Sato (17)]. The total cytochrome P450 content in the 3ml reaction mixture was determined after the addition of sodium dithionite.

RESULTS

The effects of Ca^{++} and ADP on the NADPH supported 11β-hydroxylation of DOC by a beef adrenal cortical mitochondrial preparation in 50% glycerol is shown in Table 18. Ca^{++} was found to stimulate 11β-hydroxylation in this system, a result which is in accord with the previous report by Pèron *et al.* (10) in which freshly prepared rat adrenal mitochondria were used. As anticipated from the study presented in Section III, the addition of ADP resulted in an inhibition of that Ca^{++} stimulation. It is also seen from the data in Table 18 that the storage of the mitochondrial preparation in 50% glycerol at -20°C for at least a month resulted in no change in the 11β-hydroxylase activity or in the effects of Ca^{++} or Ca^{++} plus ADP on that activity.

Mitochondria suspended in 12mM Tris, pH7.5, showed a slight increase or no change in 11β-hydroxylase activity upon storage in the frozen state at -20°C or upon repeated freezing and thawing. However, these treatments resulted in a progressive loss in the stimulation by Ca^{++} and its inhibition by ADP. The data presented in Table 19 shows that both the Ca^{++} and ADP effects on the rate of corticosterone synthesis were present when the mitochondrial preparation was preincubated with 2.0mM Na deoxycholate for 5 min. at room temperature.

In agreement with the finding by Pèron *et al.* (10), the cation stimulation of 11β-hydroxylase activity was found to be relatively specific for Ca^{++}. Ba^{++}, Mg^{++}, Zn^{++},

TABLE 18

The 11β-Hydroxylase Activity in Bovine Adrenal Mitochondrial Preparation Stored in 50% Glycerol at -20°C

Addition	Days of Storage	
	0	31
	nmole/corticosterone/mg protein/min	
-	0.85	0.95
Ca^{++}	6.80	6.30
Ca^{++} + ADP	2.55	2.88

Ca^{++} and ADP, when present, were at a final concentration of 4.17mM and 50μM respectively.

TABLE 19

The Effects of Ca^{++} and ADP on the 11β-Hydroxylase Activity in Detergent Treated Mitochondrial Preparation

Addition	ADP Present				
	0	.05mM	0.1mM	0.2mM	0.4mM
	nmoles/mg protein/min				
0.417mM Ca^{++}	3.9	3.2	2.7	1.7	1.4

The mitochondrial preparation was preincubated for 5 min. at room temperature in the presence of 2.0mM Na deoxycholate and then centrifuged at 105,000 x g for 40 min. The pellet was suspended in 12mM Tris buffer pH7.5 and 0.3mg protein was used in the incubation. Incubation conditions are described in Methods and the rate of corticosterone synthesis in the absence of Ca^{++} was 0.71 nmole/mg protein/min.

Cu^{++}, Fe^{++} and Co^{++} were without effect while, Sr^{++}, Mn^{++} and Ca^{++}, stimulated the system by a maximum of 63, 100, and 575 percent respectively in the same experiment. Furthermore, Sr^{++} was found to be a competitive inhibitor of the Ca^{++} stimulation (Fig. 6).

The data in Table 20 shows the effect of the addition of several nucleotides on the Ca^{++} stimulation of the 11β-hydroxylation of DOC. It is to be noted that the nucleoside monophosphates were essentially non-inhibitory. Among the nucleoside di and triphosphates, only ADP, CDP and ATP were found to be inhibitory with ADP being more inhibitory than the other nucleotides. An ADP and ATP concentration curve is presented in (Fig. 7), and in agreement with the data presented in Section III. ADP was found to be more inhibitory than ATP throughout the concentration range. The possibility that the ATP inhibition is the result of its hydrolysis to ADP by the action of a mitochondrial ATPase was tested. The ATP inhibition was found to be only to a small extent due to its conversion to ADP, since 0.2mM ATP resulted in a 53% inhibition while, in the presence of an ATP generating system, consisting of creatine phosphate and creatine phosphokinase, a 46% inhibition was observed. It should be noted that phosphoenol pyruvate inhibited the 11β-hydroxylase activity.

The data in Table 21 shows that the 11β-hydroxylase activity in the absence of Ca^{++} is not inhibited by ADP. EGTA was added in this experiment to assure the removal of

FIG. 6. The effect of Sr^{++} on the Ca^{++} stimulation of corticosterone synthesis. No Sr^{++}, ●; 1.67mM Sr^{++}, o; 4.17mM Sr^{++}, . K_m values are 2.3, 3.3 and 5mM respectively. V_{max} value is 11.8 nmoles/mg protein/min.

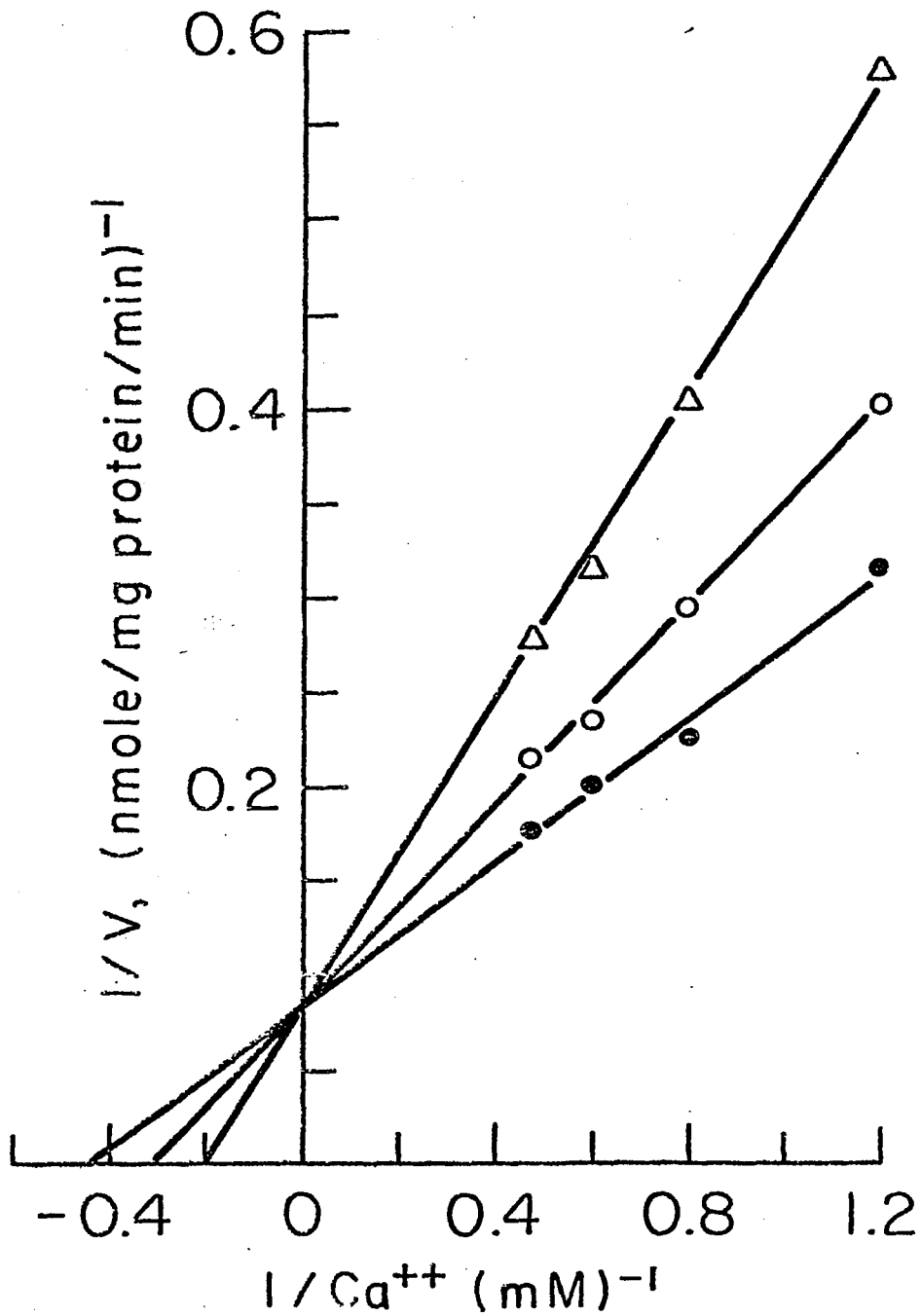
0.6
0.4
0.2
1/V, (nmole/mg protein/min)⁻¹
-0.4
0
0.4
0.8
1.2
1/Ca⁺⁺ (mM)⁻¹

TABLE 20

The Inhibition of Corticosterone Synthesis

by Various Nucleotides

Addition		Adenine	Guanine	Uracil	Cytosine
	mM	% Inhibition of Corticosterone Synthesis			
Nucleoside-5'-P	2.00	15	11	2	15
Nucleoside-5'-diP	0.05	38	0	3	16
"	0.20	66	0	3	47
Nucleoside-5'-triP	0.05	16	0	6	9
"	0.20	44	3	19	13

The data presented are those from two experiments. In one experiment the nucleoside-5'-P compounds were tested, and in the second experiment the nucleoside-5'-diP and nucleoside-5'-triP compounds were tested. The rate of corticosterone synthesis, in the absence of the nucleotides, was 5.3 nmoles/mg protein/min for the first experiment and 7.0 nmoles/mg protein/min for the second experiment. Ca^{++} was present at a final concentration of 4.17mM in all cases.

FIG. 7. The effect of ADP and ATP on the rate of corticosterone synthesis in the presence of 4.17mM Ca^{++}.

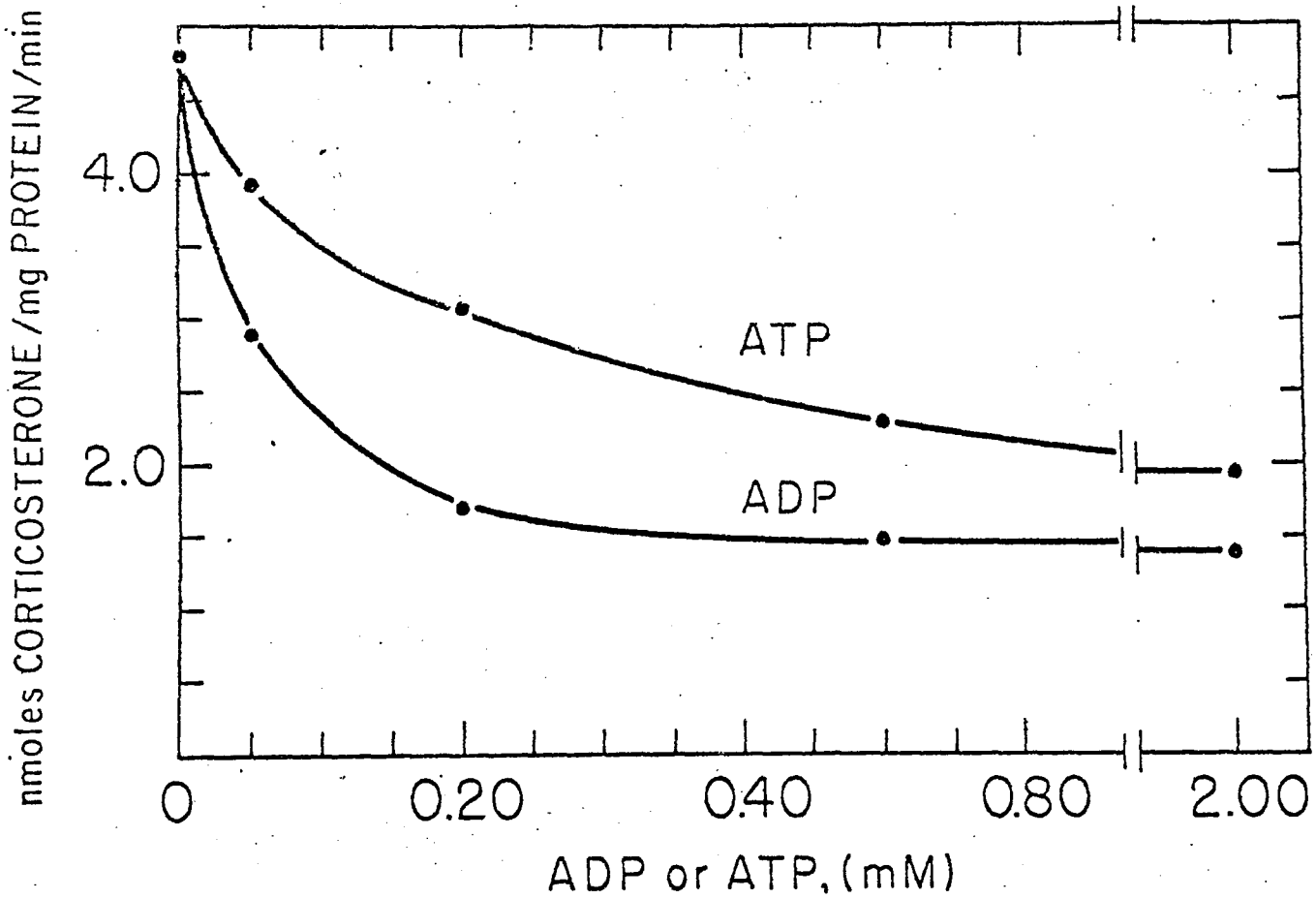
nmoles CORTICOSTERONE /mg PROTEIN/min
4.0
2.0
ATP
ADP
0
0.20
0.40
0.80
2.00
ADP or ATP, (mM)

TABLE 21

The Effect of ADP on Corticosterone Synthesis in the Absence of Ca^{++}

Addition	Corticosterone Found
	nmoles/mg protein/min
-	0.81
ADP	0.89
EGTA	1.04
EGTA + ADP	0.96

Mitochondrial preparation in which the glycerol was removed was used in this experiment (0.17mg). ADP and EGTA, when present, were at a final concentration of 2.0mM and 16.7mM respectively.

any endogenous Ca^{++}.

The Ca^{++} stimulated 11β-hydroxylase activity was found to have a pH optimum between pH7.0 and 7.5 (Fig. 8). The same pH optimum range was also found in the presence of Ca^{++} plus 2mM ADP and the ADP inhibition was constant (65%) throughout the entire pH range.

The effects of Ca^{++} and ADP on the individual steps in the 11β-hydroxylase sequence of reactions were tested. The binding of ^{14}C-DOC to the mitochondrial preparation was studied, and as seen from the data in Table 22, no effect on either the binding constant (K_S) or the maximum amount of DOC specifically bound (13) to the mitochondrial protein, was seen in the presence of concentrations as high as 5.0mM Ca^{++} or 3.0mM ADP. The type I difference spectra induced by the addition of DOC to the mitochondrial preparation, which is considered to be a measure of the binding of DOC to the hydroxylase cytochrome P450 (18) was studied. In agreement with the ^{14}C-DOC binding data, the magnitude of this spectral change at different concentrations of DOC was not changed in the presence of either Ca^{++} or ADP (Table 23).

The hydroxylase electron transferring chain consists of at least three protein components (19,20). A flavoprotein (NADPH diaphorase) catalyzes the transfer of electrons from NADPH to the second electron carrier, a non-heme iron protein (adrenodoxin). This, in turn, reduces the third component, cytochrome P450. In the presence of CO, the reduction rate of the overall chain by NADPH was measured by monitoring the

FIG. 8. The effect of pH on the 11β-hydroxylation of DOC. MES buffer, ●; MOPS buffer, l; Tris buffer, Δ. Ca^{++} was present in all conditions at a final concentration of 4.17mM.

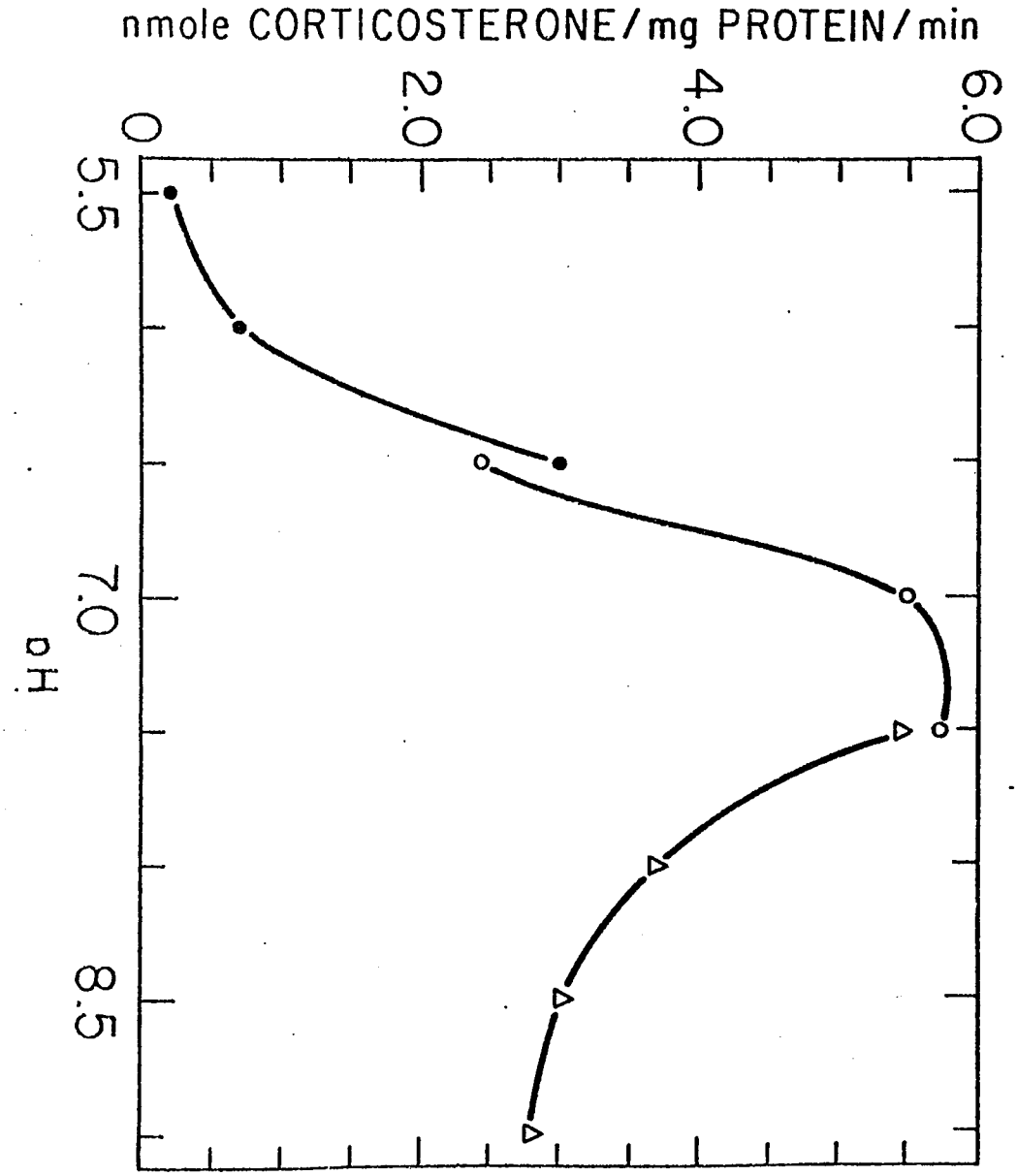
nmole CORTICOSTERONE/mg PROTEIN/min
0
2.0
4.0
6.0
5.5
7.0
8.5
pH

TABLE 22

The Effect of Ca^{++} and ADP on Deoxycorticosterone Binding to the Mitochondrial Preparation

Experiment	Addition	K_s M x 10^6	DOC Bound nmole/mg protein
I	-	0.71	0.45
I	Ca^{++}	0.74	0.45
II	-	0.72	0.48
II	ADP	0.77	0.48

In Experiment I protein concentration was 1.55mg/2.0ml of incubation medium and in II it was 0.85mg/2.0ml. Ca^{++} was at a final concentration of 5.0mM and ADP at a final concentration of 3.0mM.

TABLE 23

The Effect of ADP and Ca^{++} on DOC Induced Type I Difference Spectra

Addition	DOC Added			
	1.5μM	3.0μM	4.5μM	6.0μM
		O.D. Δ(385-420nm)		
-	0.031	0.041	0.049	0.052
ADP	0.029	0.045	0.051	0.056
Ca^{++}	0.028	0.042	0.048	0.052

Each cuvette contained 2.6mg of mitochondrial protein. ADP and Ca^{++}, when added, were at a final concentration of 2.0mM and 3.3mM respectively. The final volume in each cuvette was brought up to 3.0ml by the addition of a 12mM tris solution at pH7.5. DOC was added to the sample cuvette at the indicated concentrations and an equal volume of ethanol were added to the reference cuvette.

appearance of the reduced P450-CO complex at 450nm. It is seen from the data in Table 24 and (Fig. 9) that Ca^{++} stimulated this reduction rate and that the Ca^{++} stimulation was inhibited by ADP but not AMP. It is apparent that these effects are independent of the presence or absence of DOC. It is also seen from the data in Table 24 (experiment II) that in the presence of Sr^{++} the rate of cytochrome P450 reduction was increased to only one fourth of that obtained by Ca^{++}.

The overall reduction reaction of cytochrome P450 was dissected, by the use of artificial electron acceptors, into the NADPH diaphorase segment, which measures the flavoprotein activity and the NADPH-cytochrome c reductase activity which involves both adrenodoxin and the flavoprotein (21). Both, NADPH diaphorase and NADPH-cytochrome c reductase activities proceeded at higher rates than the rate of corticosterone synthesis measured in the same preparation (Table 25). The presence of Ca^{++} has no effect on either reduction rate. Some inhibition of NADPH diaphorase and NADPH-cytochrome c reductase activities was present at the higher ADP concentrations. This inhibition does not correspond to the ADP effect on corticosterone synthesis, however, since in the presence of 0.25mM ADP there was no inhibition of the cytochrome c reductase activity, while the Ca^{++} stimulated corticosterone synthesis was 80% inhibited.

The kinetics of the Ca^{++} stimulation and ADP inhibition are shown in Figs. 10,11, and 12. It is seen that the ADP

FIG. 9. Semilogarithmic plot of the rate of cytochrome P450 reduction. When present Ca^{++} and DOC were at 0.67mM and 30μM final concentrations respectively. DOC was added in 5μl of ethanol and an equal volume of ethanol was used in cuvettes where DOC was not added.

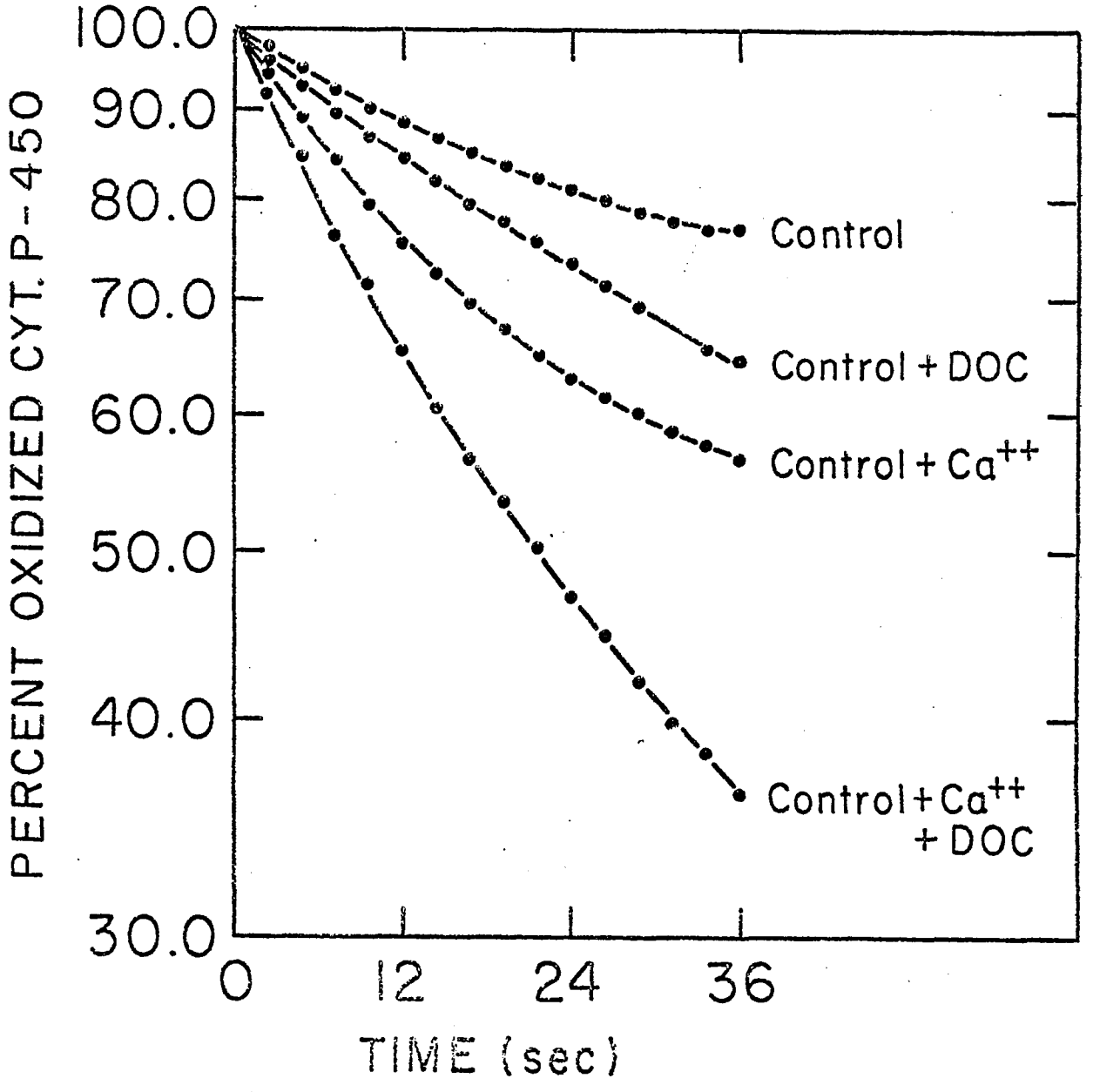
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
PERCENT OXIDIZED CYT. P-450
0
12
24
36
TIME (sec)
Control
Control + DOC
Control + Ca++
Control + Ca++ + DOC

TABLE 24

Effects of Ca^{++} and ADP on the Rate of Cytochrome P450 Reduction

Experiment	Additions	nmole cyt.P450 reduced/mg protein/min		% Change	
		-	DOC	-	DOC
I	-	1.2	1.4	-	-
I	Ca^{++}	3.4	4.6	+183	+228
I	Ca^{++} + AMP	3.8	5.6	+216	+300
I	Ca^{++} + ADP	1.9	2.3	+ 58	+ 64
II	-	1.9	2.1	-	-
II	Ca^{++}	9.5	10.8	+400	+414
II	Sr^{++}	3.9	3.9	+105	+ 86
II	Ca^{++} + ADP	1.9	2.1	0	0

Experiment I; when present, Ca^{++}, AMP, and ADP were at a final concentration of 0.67mM, 2.0mM and 2.0mM respectively and in experiment II Ca^{++}, Sr^{++} and ADP were at a final concentration of 4.17mM, 4.17mM and 2.0mM respectively. DOC, when added, was at a final concentration of 30μM.

TABLE 25

The Effect of ADP on NADPH-Cytochrome c Reductase, NADPH Diaphorase and Steroid 11β-Hydroxylase Activities In The Mitochondrial Preparation

	Addition	ADP Present			
		0	0.25mM	0.50mM	2.50mM
		nmole/mg protein/min			
NADPH-cyt.c reductase	-	50.5	50.5	47.9	36.5
"	Ca^{++}	56.1	53.3	40.7	33.7
NADPH-diaphorase	-	54.0	-	-	41.0
"	Ca^{++}	59.6	-	-	46.1
Steroid 11β-hydroxylase	-	2.5	-	-	-
"	Ca^{++}	13.1	2.5	2.5	2.3

The final concentration of Ca^{++}, when present, was 1.67mM.

inhibition with respect to Ca^{++} is non-competitive as is the ADP inhibition with respect to DOC. The Ca^{++} stimulation with respect to DOC shows an uncompetitive type of stimulation.

Additional information on the interactions between Ca^{++} and ADP in their effect on the 11β-hydroxylase system was obtained by investigating the effects of preincubation of the mitochondrial preparation with Ca^{++} or ADP or Ca^{++} plus ADP (Table 26). It is seen that a preincubation with Ca^{++} results in a sharp decrease in the ADP inhibition. On the other hand, when the preincubation is carried out in the presence of ADP, the inhibition is increased. When both Ca^{++} and ADP are present during the preincubation, the ADP inhibition is decreased but to a much lesser extent than when only Ca^{++} is present during the preincubation.

DISCUSSION

The stimulation by Ca^{++} of the 11β-hydroxylation of DOC to form corticosterone in the beef adrenal cortical mitochondrial preparation used, has been found to be inhibited by ADP. The data presented in Tables 19 and 25 eliminate an entry of NADPH into the mitochondria as a factor in these Ca^{++} and ADP effects. Thus, NADPH diaphorase and NADPH-cytochrome c reductase activities, the first and the second reduction reactions in the steroid hydroxylase electron transporting chain (21) were not influenced by the presence of Ca^{++} (Table 25). In addition, at an ADP concentration

FIG. 10. The inhibition by ADP, with respect to Ca^{++}, of the 11β-hydroxylation of DOC. No ADP, ●; 1 x 10^{-5}M ADP, o; 5 x 10^{-5}M ADP, Δ. V_{max} values are 13.3, 10.0 and 5.0 nmoles/mg protein/min respectively and K_m value is 2.9mM.

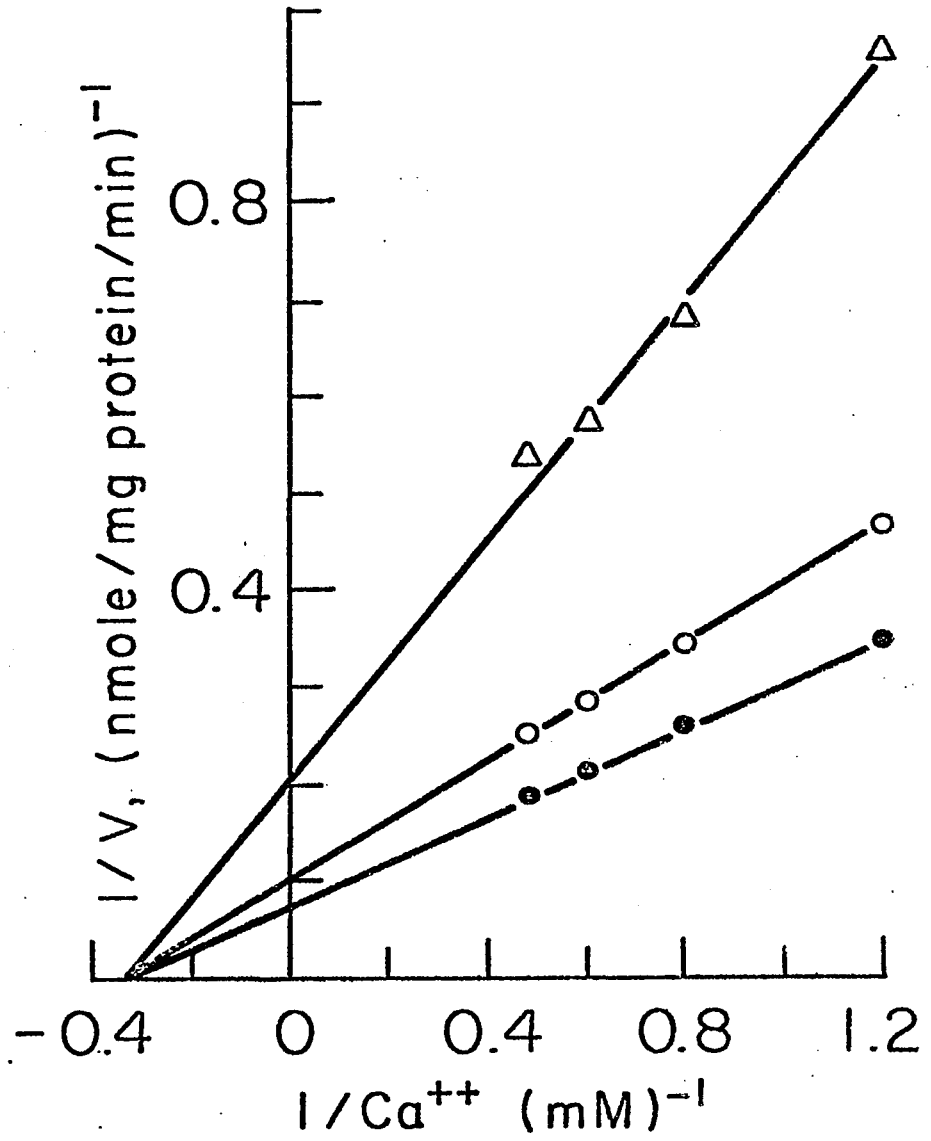
1/V, (nmole/mg protein/min)$^{-1}$
0.8
0.4
-0.4
0
0.4
0.8
1.2
1/Ca^{++} (mM)$^{-1}$

FIG. 11. The inhibition by ADP, with respect to DOC, of the Ca^{++} stimulated 11β-hydroxylation of DOC. No ADP, ●; 2 x 10^{-4}M ADP, o; V_{max} values are 3.7 and 2.1 nmoles/mg protein/min respectively and K_m value is 2.9 x 10^{-6}M. Ca^{++} was present at a final concentration of 0.417mM.

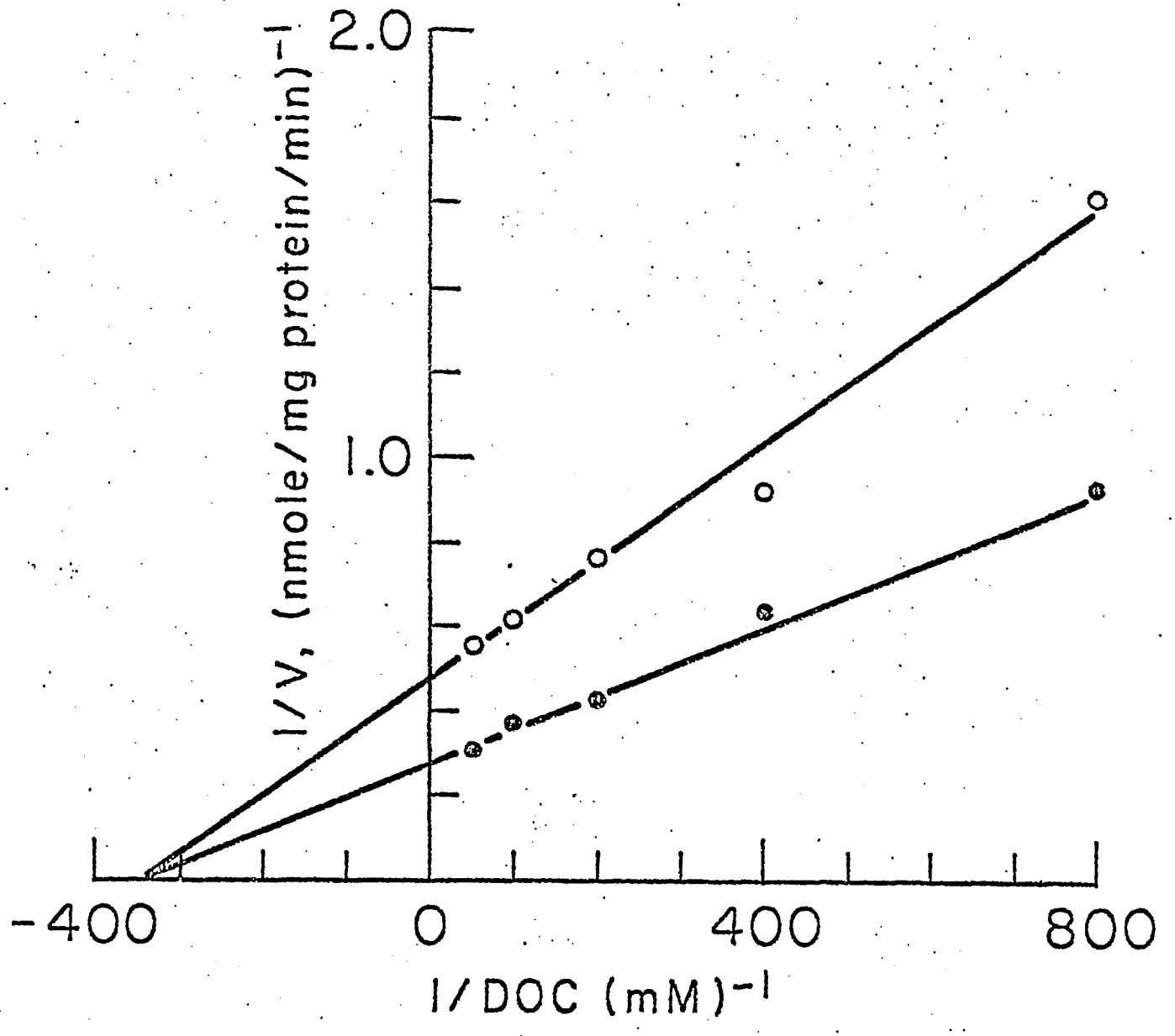
2.0
1.0
1/V, (nmole/mg protein/min)$^{-1}$
-400
0
400
800
1/DOC (mM)$^{-1}$

FIG. 12. The Ca^{++} stimulation, with respect to DOC, of 11β-hydroxylation of DOC. 0.833mM Ca^{++}, ●; 0.417mM Ca^{++}, o; V_{max} values are 5.6 and 2.7 nmoles/mg protein/min respectively and K_m values are 6.7 and 3.4 x 10^{-6}M respectively.

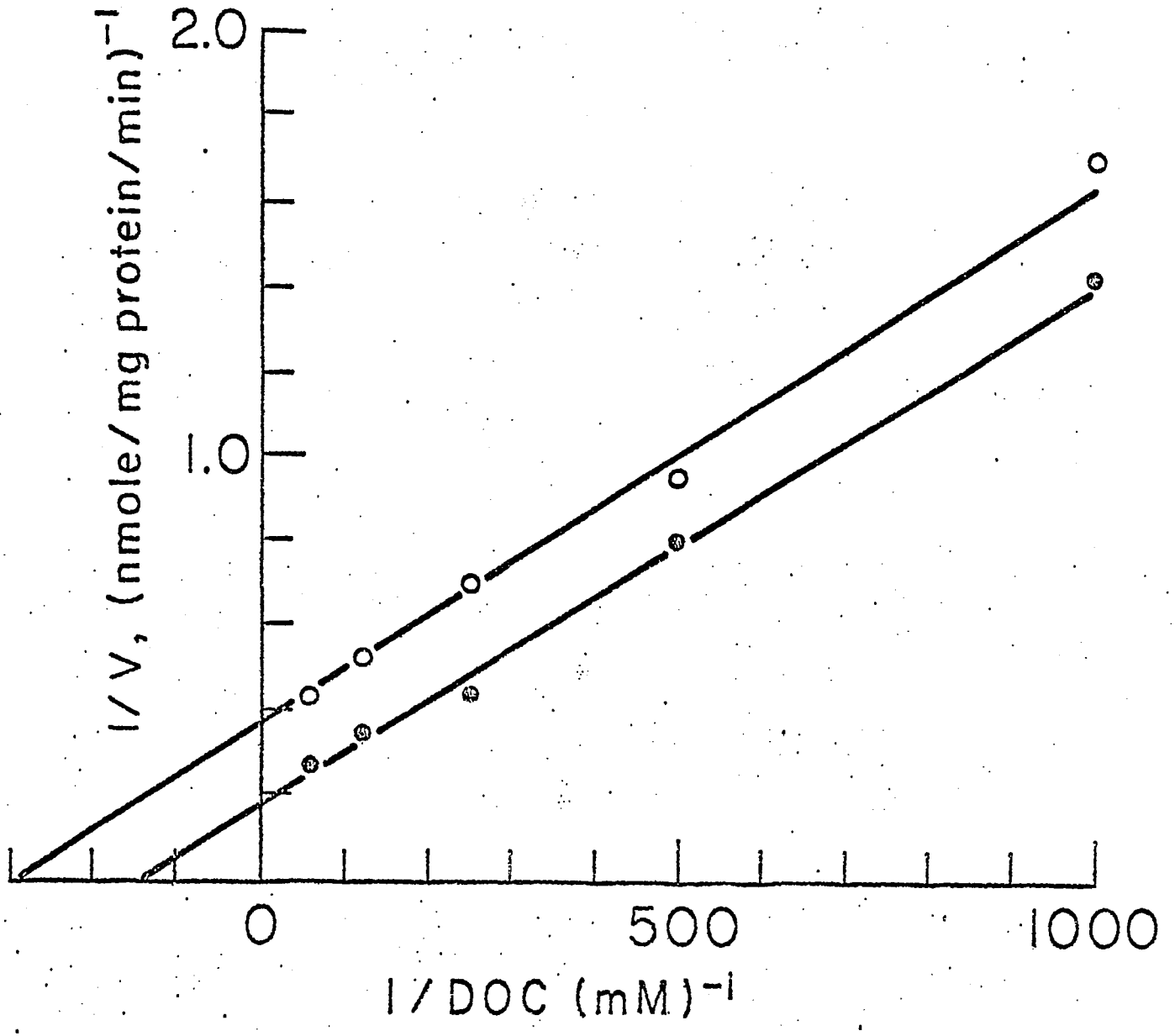
2.0
1.0
1/V, (nmole/mg protein/min)$^{-1}$
0
500
1000
1/DOC (mM)$^{-1}$

TABLE 26

The Effect of Preincubation in the Presence of Ca^{++}, ADP and Ca^{++} + ADP on the Inhibition of 11β-Hydroxylase by ADP

ADP µM	Preincubation Conditions I Rate	I %Inhibition	II Rate	II %Inhibition	III Rate	III %Inhibition
0	8.6	-	9.2	-	9.0	-
50	7.7	10	1.6	83	5.6	38
100	7.6	12	1.5	84	3.5	61

The mitochondrial preparation, buffer and NADPH were preincubated for 10 min. under the following conditions. I, Ca^{++} was present during the preincubation. The indicated concentrations of ADP were then added and the reaction was started with DOC. II, the indicated concentrations of ADP were present during the preincubation. Ca^{++} was then added and the reaction started with DOC. III, 50 or 100µM ADP plus Ca^{++} were present during the preincubation and then the reaction was started with DOC. When ADP was not present in condition III, Ca^{++} was added after the preincubation and the reaction was also started with DOC. The final concentration of Ca^{++} in all cases was 4.17mM. The rate is expressed as nmoles corticosterone formed/mg protein/min. When no preincubations were involved 50µM ADP inhibited by 56% and 100µM ADP inhibited by 73%.

which gave about 80% inhibition of the Ca^{++} stimulated rate of corticosterone synthesis, no effect was seen on the reduction of NADPH-cytochrome c reductase. Furthermore, both the Ca^{++} and the ADP effects were present when the mitochondria were disrupted (as seen by electron microscopy) by the use of Na deoxycholate (Table 19). In agreement with the results reported by Pèron et al. (10) both Mn^{++} and Sr^{++} stimulated 11β-hydroxylation but to a much lesser extent than Ca^{++}. The finding that Sr^{++} acted as a competitive inhibitor for the Ca^{++} stimulation (Fig. 6) indicates that both cations act at the same site. Other than ADP, only ATP and CDP of the 12 nucleotides tested showed significant inhibition, and at the 0.05mM level, were less than half as effective as ADP (Table 20, Fig. 7). None of the non-inhibitory nucleotides could reverse the ADP inhibition. In addition, adenosine phosphosulfate, 3',5'-ADP, adenine imidophosphate and adenylmethylene diphosphate at 0.2mM were not inhibitory and did not effect the ADP inhibition.

When individual steps of the hydroxylase sequence of reaction were tested, it was found that neither the binding of DOC to cytochrome P450, as measured directly (Table 22) or by difference spectroscopy (Table 23), nor the NADPH diaphorase or NADPH-cytochrome c reductase activities (Table 25) were involved in the Ca^{++} and ADP effects. However, the NADPH-cytochrome P450 reductase activity, which requires the presence of the three protein components of the hydroxy-

lase electron transferring system (21), was stimulated by Ca^{++} and this Ca^{++} stimulation was inhibited by ADP. AMP which has been shown to have no effect on 11β-hydroxylation (Table 20), also does not effect the reduction of P450 (Table 24). In accord with their effects on corticosterone synthesis, Ca^{++} has a greater effect on P450 reduction than does Sr^{++} (Table 24). These data strongly suggest that the effect of both Ca^{++} and ADP on the synthesis of corticosterone are localized at the adrenodoxin-cytochrome P450 reduction step. A regulation of 11β-hydroxylase system at this step is consistent with the report by Harding et al. (22) that the reduction of P450 appears to be rate-limiting in 11β-hydroxylation of DOC, a conclusion consistent with the data obtained by Baron et al. (23), and the data presented in Table 25.

ADP was found to have no effect on 11β-hydroxylation of DOC in the absence of Ca^{++} (Table 21). This suggests an interaction between the effects of ADP and Ca^{++} in a regulation of this enzymatic activity. An understanding of such an interaction was gained by the experiment reported in Table 26. It is seen that Ca^{++} stimulated corticosterone synthesis to the same extent whether it was present during the preincubation or only in the final incubation. However, a preincubation of the mitochondrial preparation with Ca^{++} resulted in a sharp decrease in the ADP inhibition (from 56% and 73% to 10% and 12% at 50 and 100μM ADP respectively). This indicates that a preexposure of the

hydroxylase system to Ca^{++} modifies the magnitude of the ADP effect. Similarly, when the mitochondrial preparation was preincubated with ADP, the ADP inhibition was increased (from 56% and 73% to 83% and 84% at 50 and 100μM ADP respectively). This indicates that an interaction between the hydroxylase system and ADP occurs in the absence of Ca^{++}, although ADP has no effect on the non-stimulated rate of corticosterone synthesis (Table 21). It is to be noted that the extent of the ADP interaction with this system appears to be a function of the presence of Ca^{++} since, when ADP plus Ca^{++} were present, the ADP inhibition is less than when only ADP was present during the preincubation (38% and 61% vs. 83% and 84% at 50 and 100μM respectively). It would then appear that a reciprocal relationship between ADP and Ca^{++} is present in which both modifiers effect the action of the other. In addition, it is not likely that the ADP inhibition is a simple reversal of the Ca^{++} effect since the Ca^{++} stimulation of corticosterone synthesis was found to be uncompetitive in nature with respect to DOC (Fig. 12), while ADP was found to be a non-competitive inhibitor, also with respect to DOC, in the Ca^{++} stimulated system (Fig. 11). In addition, ADP was found to be a noncompetitive inhibitor of the Ca^{++} stimulation suggesting two different sites for the action of both modifiers (Fig. 10).

In view of these data, it is suggested that ADP and Ca^{++} influence the relationship between adrenodoxin and

cytochrome P450 in such a way that in the presence of Ca^{++} an active cytochrome P450 adrenodoxin complex is formed and a less active complex is formed in the presence of Ca^{++} plus ADP. The formation of this active complex is manifested in an accelerated rate of electron transfer from adrenodoxin to cytochrome P450. Whether the increased hydroxylation rate is a function only of the increase of cytochrome P450 reduction rate is not known. Evidence for the formation of such adrenodoxin cytochrome P450 complex as an essential step in camphor hydroxylation in a bacterial system has been presented by the work of Gunsalus et al. (24). Furthermore, an essential and specific role for adrenodoxin in a reconstituted system for the 11β-hydroxylation of DOC has been described (25,26).

When mitochondria suspended in 12mM Tris buffer, pH7.5, were stored in the frozen state, the basal 11β-hydroxylase activity remained unchanged but the Ca^{++} and ADP effects were progressively reduced with time of storage. Preliminary experiments with a partially purified system indicated that the effects of both Ca^{++} and ADP could be modified or eliminated. Both of these observations indicate the involvement of as yet unknown factor in this aspect of the 11β-hydroxylase system. A possible factor may be phospholipids which have been found to stimulate 11β-hydroxylase activity in an acetone powder preparation of beef mitochondria (27).

The data presented in this report indicates that the control of the 11β-hydroxylation of DOC in adrenal mitochondria resides at the adrenodoxin-cytochrome P450 reduction step and that the Ca^{++} stimulation of corticosterone synthesis and its inhibition by ADP is the function of an interaction between Ca^{++} and ADP with either cytochrome P450 or adrenodoxin or both.

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APPENDIX

ACTH	adrenocorticotrophic hormone
aminoglutethimide	α-ethyl-p-aminophenyl-glutarimide
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
cAMP	adenosine 3',5'-cyclic-monophosphate
CDP	cytosine 5'-diphosphate
°C	degree centrigrade
cyanoketone	2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androstene-3-one
cm	centimeter
EDTA	ethylenedinitrilotetraacetic acid
EGTA	ethyleneglycol-bis(β-aminoethyl ether)N,N'-tetraacetic acid
E.P.R.	electron paramagnetic resonance
g =	force of gravitation
g,mg,µg =	gram, milligram, microgram
M, mM, µM =	molar, millimolar, micromolar
m-,µ-,n- =	milli-, micro-,nano-
min =	minutes
ml, µl =	milliter, microliter
MES	2[N-morpholino]ethane sulfonic acid
MOPS	morpholinopropane sulfonic acid
metopirone	2-methyl-1,2-di(3-pyridyl)-1-propanone
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotainmide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate

APPENDIX (Con't.)

NADPH	reduced nicotinamide adenine dinucleotide phosphate
nmoles	nanomoles
nM	nanomolar
nm	nanometer
PEP	phosphoenolpyruvate
pmoles	picomoles
RNA	ribonucleic acid
S	spin quantum number
S.D.S.	sodium dodecylsulfate
Tris	tris(hydroxymethyl)aminomethane
µl	microliter
µM	micromolar