STUDIES ON THE 11β-HYDROXYLATION OF 
DEOXYCORTICOSTERONE

by

DENIS GEORGE WILLIAMSON
B.Sc., University of British Columbia, 1963

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in the Department of
Biochemistry

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA
May, 1968
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Biochemistry

The University of British Columbia
Vancouver 8, Canada

Date May 27, 1968
ABSTRACT

Steroid hydroxylases are members of a group of enzymes termed "mixed function oxidases". These enzymes catalyze the introduction of an atom of molecular oxygen into the substrate molecule concomitant with the oxidation of NADPH. This thesis describes studies carried out with a steroid 11β-hydroxylase prepared from an acetone powder of bovine adrenal mitochondria. The conversion of deoxycorticosterone to corticosterone has been employed to measure the 11β-hydroxylase activity. The modes of action of two inhibitors of the 11β-hydroxylation reaction, namely, dicumarol and Metopirone have been examined as a means of obtaining information concerning the mechanism of 11β-hydroxylation.

Kinetic examination of dicumarol inhibition of 11β-hydroxylation indicated that this compound had at least two inhibitory actions. At concentrations below 100 μmoles/l, dicumarol was a noncompetitive inhibitor of 11β-hydroxylation. At dicumarol concentrations above 100 μmoles/l a second inhibitory action became apparent. This second inhibition could be greatly diminished by increasing the substrate concentration.

Kinetic examination of Metopirone inhibition of 11β-hydroxylation indicated that this compound was a competitive inhibitor of the 11β-hydroxylase reaction. In addition Metopirone had a higher affinity for the 11β-hydroxylase system than did substrate deoxycorticosterone. The $K_i$ for Metopirone was $1.0 \times 10^{-7}$ moles/l while the $K_m$ for deoxycorticosterone was $5.5 \times 10^{-6}$ moles/l.
Cytochrome P-450 has been shown to be both the oxygen-activating and substrate binding component of steroid hydroxylases. The interactions of steroid substrate, dicumarol, and Metopirone with this hemoprotein were therefore examined. The ability of cytochrome P-450 to bind carbon monoxide forming a complex exhibiting an absorption maximum at 450 μm was employed to measure this cytochrome in the 11β-hydroxylase preparation.

Cytochrome P-450 present in the mitochondrial acetone powder preparation was found to be unstable, undergoing spontaneous decomposition at temperatures above 30°C to cytochrome P-420, a hemoprotein that does not function in 11β-hydroxylation. However, the rate and extent of cytochrome P-450 decomposition was diminished by the addition of steroid substrate, suggesting that substrate was binding to and stabilizing the hemoprotein. A similar stabilization of cytochrome P-450 was produced upon addition of Metopirone and of low concentrations of dicumarol. Hence these inhibitors could also bind to cytochrome P-450. Dicumarol at high concentrations enhanced the rate of breakdown of cytochrome P-450 to cytochrome P-420. Thus this compound had two opposing effects on cytochrome P-450.

The binding of deoxycorticosterone to cytochrome P-450 resulted in spectral changes in the hemoprotein that could be measured by the technique of difference spectrophotometry. The substrate concentration required for half-maximal spectral change, and hence half-maximal binding to cytochrome
P-450 was almost identical to its $K_m$ for 11β-hydroxylation. The deoxycorticosterone-induced spectral change in cytochrome P-450 was diminished by addition of Metopirone or by high concentrations of dicumarol but not by low concentrations of dicumarol.

Metopirone inhibits 11β-hydroxylation by binding to cytochrome P-450 and preventing the concomitant binding of substrate deoxycorticosterone. The binding of Metopirone and deoxycorticosterone to cytochrome P-450 is competitive in nature, hence competitive kinetics are observed with Metopirone inhibition of the 11β-hydroxylation reaction.

Dicumarol exerts two inhibitory actions on 11β-hydroxylation. At low concentrations this compound binds to cytochrome P-450 but does not affect substrate binding to the heme-protein, resulting in noncompetitive inhibition of 11β-hydroxylation. The binding of dicumarol at these concentrations therefore must inhibit the interaction of steroid substrate and oxygen to diminish the rate of 11β-hydroxylation. Dicumarol, at high concentrations, inhibits the binding of deoxycorticosterone to cytochrome P-450 thus producing its second inhibitory action on 11β-hydroxylation. In addition, binding of dicumarol at high concentrations results in the breakdown of cytochrome P-450.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>i</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>PUBLICATIONS</td>
<td>xiv</td>
</tr>
<tr>
<td>ABBREVIATIONS USED</td>
<td>xv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xvi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Steroid Hydroxylases</td>
<td>2</td>
</tr>
<tr>
<td>Steroid 11β-Hydroxylase</td>
<td>2</td>
</tr>
<tr>
<td>Components of the 11β-Hydroxylase</td>
<td>6</td>
</tr>
<tr>
<td>NADPH Reductase</td>
<td>8</td>
</tr>
<tr>
<td>Nonheme Iron Protein</td>
<td>8</td>
</tr>
<tr>
<td>Cytochrome P-450</td>
<td>9</td>
</tr>
<tr>
<td>Cytochrome P-420</td>
<td>13</td>
</tr>
<tr>
<td>Cytochrome P-450 as the Substrate Binding Site</td>
<td>14</td>
</tr>
<tr>
<td>MATERIALS and METHODS</td>
<td>19</td>
</tr>
<tr>
<td>(a) Solvents</td>
<td>19</td>
</tr>
<tr>
<td>(b) Chemicals</td>
<td>19</td>
</tr>
<tr>
<td>(c) Steroids</td>
<td>20</td>
</tr>
<tr>
<td>(d) Chromatography Procedures</td>
<td>20</td>
</tr>
<tr>
<td>(e) Isolation of the 11β-Hydroxylase from Beef Adrenal Glands</td>
<td>21</td>
</tr>
<tr>
<td>(i) Preparation of beef adrenal mitochondria</td>
<td>21</td>
</tr>
<tr>
<td>(ii) Preparation of the mitochondrial acetone powder</td>
<td>21</td>
</tr>
<tr>
<td>(iii) Preparation of the 11β-hydroxylase from sonicated adrenal mitochondria</td>
<td>22</td>
</tr>
<tr>
<td>(f) Protein Determinations</td>
<td>23</td>
</tr>
<tr>
<td>(g) General Incubation Conditions</td>
<td>23</td>
</tr>
<tr>
<td>(h) Studies on NADPH Oxidation</td>
<td>24</td>
</tr>
<tr>
<td>(i) Studies on Corticosterone Formation (11β-Hydroxylation)</td>
<td>25</td>
</tr>
<tr>
<td>(i) Kinetic studies on 11β-hydroxylation</td>
<td>26</td>
</tr>
<tr>
<td>(ii) Isolation, purification, and measurement of deoxycorticosterone and corticosterone</td>
<td>26</td>
</tr>
<tr>
<td>(iii) Radioassay method</td>
<td>29</td>
</tr>
<tr>
<td>(j) Spectrophotometric Analysis of the Enzyme Preparation</td>
<td>29</td>
</tr>
<tr>
<td>(k) Ammonium Sulfate Fractionation of the Enzyme Preparation</td>
<td>30</td>
</tr>
<tr>
<td>(l) Studies on Cytochromes P-450 and P-420</td>
<td>31</td>
</tr>
<tr>
<td>(i) Effects of 11β-hydroxylase substrates, inhibitors (Dicumarol or Metopirone), and incubation conditions</td>
<td>31</td>
</tr>
<tr>
<td>(ii) Substrate and inhibitor-induced spectral changes in cytochrome P-450</td>
<td>33</td>
</tr>
<tr>
<td>(m) Studies on Bovine Adrenal Mitochondrial Lipids</td>
<td>35</td>
</tr>
<tr>
<td>(i) Acetone-extracted lipid fraction</td>
<td>35</td>
</tr>
<tr>
<td>(ii) Preliminary fractionation of the acetone-extracted lipid material</td>
<td>35</td>
</tr>
<tr>
<td>(iii) Extraction of the total lipids of adrenal mitochondria</td>
<td>36</td>
</tr>
<tr>
<td>(iv) Silica gel column chromatography of the lipid fraction soluble in ethanol at -20°C</td>
<td>38</td>
</tr>
<tr>
<td>(v) Saponification of the adrenal mitochondrial acetone powder</td>
<td>39</td>
</tr>
</tbody>
</table>
PART I GENERAL PROPERTIES OF THE 11β-HYDROXYLASE EXTRACTED FROM A BOVINE ADRENAL MITOCHONDRIAL ACETONE POWDER

RESULTS

(a) NADPH Requirement of the 11β-Hydroxylase

(b) Kinetic Study of 11β-Hydroxylation

(c) Spectrophotometric Studies on the Enzyme Preparation
   (i) Reduced-oxidized and carbon monoxide difference spectra of the enzyme preparation
   (ii) Ammonium sulfate fractionation of the enzyme preparation

(d) 11β-Hydroxylase from Sonicated Adrenal Mitochondria
   (i) NADPH oxidation and 11β-hydroxylation by the sonicated adrenal mitochondria preparation
   (ii) Effect of acetone on the sonicated enzyme preparation

(e) Studies on Cytochrome P-450 in the Adrenal Mitochondrial Acetone Powder Preparation
   (i) Effect of incubation time and temperature on cytochromes P-450 and P-420
   (ii) Effect of steroid substrates on cytochromes P-450 and P-420

DISCUSSION

PART II THE ROLE OF QUINONES IN 11β-HYDROXYLATION

RESULTS

(a) Effect of Acetone-Extracted Lipid and Menadione (Vitamin K₃) on NADPH Oxidation

(b) Effect of Acetone-Extracted Lipid and Menadione on 11β-Hydroxylation
(c) A Possible Site for Menadione Reduction 108

(d) Examination of the Acetone-Extracted Lipid Factors Affecting NADPH Oxidation and 11β-Hydroxylation 113

(e) Stimulation of 11β-Hydroxylation by the Acetone-Extracted Lipid Fraction and by Asolectin 122

DISCUSSION 134

PART III DICUMAROL AND METOPIRONE INHIBITION OF 11β-HYDROXYLATION 142

RESULTS 142

(a) Inhibition of 11β-Hydroxylation by Dicumarol 142
(b) Inhibition of 11β-Hydroxylation by Metopirone 149

DISCUSSION

PART IV EFFECT OF DICUMAROL AND METOPIRONE ON CYTOCHROMES P-450 AND P-420 164

RESULTS 164

(a) Effect of Dicumarol on Cytochromes P-450 and P-420 164
(b) Effect of Metopirone on Cytochromes P-450 and P-420 179
(c) Substrate and Inhibitor-Induced Difference Spectra of Cytochrome P-450 189
   (i) Substrate-induced difference spectrum of cytochrome P-450 191
   (ii) Effect of dicumarol on the substrate-induced difference spectrum of cytochrome P-450 193
   (iii) Metopirone-induced difference spectrum of cytochrome P-450 200
   (iv) Effect of Metopirone on the substrate-induced difference spectrum of cytochrome P-450 202

DISCUSSION 205

BIBLIOGRAPHY 222
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Effect of increasing quantities of NADPH on the 11β-hydroxylation of deoxycorticosterone</td>
<td>42</td>
</tr>
<tr>
<td>II</td>
<td>11β-hydroxylation of deoxycorticosterone as a function of time</td>
<td>46</td>
</tr>
<tr>
<td>III</td>
<td>Effect of inhibitors on NADPH oxidation</td>
<td>48</td>
</tr>
<tr>
<td>IV</td>
<td>Effect of inhibitors on the 11β-hydroxylation of deoxycorticosterone</td>
<td>50</td>
</tr>
<tr>
<td>V</td>
<td>Comparison of the 11β-hydroxylase activity and cytochrome P-450 content in the enzyme systems prepared by extraction of an adrenal mitochondrial acetone powder and by ultracentrifugation of sonicated adrenal mitochondria</td>
<td>70</td>
</tr>
<tr>
<td>VI</td>
<td>Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation incubated in the presence or absence of NADPH</td>
<td>79</td>
</tr>
<tr>
<td>VII</td>
<td>Effect of mitochondrial acetone-extracted lipid, menadione, and dicumarol on the initial rate of NADPH oxidation</td>
<td>102</td>
</tr>
<tr>
<td>VIII</td>
<td>The relationship between oxidation of NADPH and reduction of cytochromes P-450 and P-420 in the 20-40% and 40-80% ammonium sulfate fractions of the mitochondrial acetone powder enzyme preparation</td>
<td>110</td>
</tr>
<tr>
<td>IX</td>
<td>Effect of adrenal mitochondria lipid fractions on the 11β-hydroxylation of deoxycorticosterone and on NADPH oxidation</td>
<td>115</td>
</tr>
<tr>
<td>X</td>
<td>Effect of adrenal mitochondria lipid fractions on NADPH oxidation</td>
<td>118</td>
</tr>
<tr>
<td>XI</td>
<td>Effect of silica gel column eluates on NADPH oxidation</td>
<td>120</td>
</tr>
<tr>
<td>XII</td>
<td>Effect of dicumarol on the 11β-hydroxylase activity, cytochrome P-450, and cytochrome P-420 content of the enzyme system prepared by sonication of adrenal mitochondria</td>
<td>178</td>
</tr>
<tr>
<td>XIII</td>
<td>Effect of Metopirone on cytochromes P-450 and P-420 in the enzyme system prepared by sonication of adrenal mitochondria</td>
<td>190</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Scheme proposed by Omura et al. for the electron transport pathway in hydroxylation reactions</td>
<td>7</td>
</tr>
<tr>
<td>2.</td>
<td>The transformation of deoxycorticosterone to corticosterone</td>
<td>18</td>
</tr>
<tr>
<td>3.</td>
<td>Structural formulae of dicumarol and Metopirone</td>
<td>18</td>
</tr>
<tr>
<td>4.</td>
<td>Oxidation of NADPH in the presence and absence of deoxycorticosterone</td>
<td>45</td>
</tr>
<tr>
<td>5.</td>
<td>The rate of 11β-hydroxylation as a function of deoxycorticosterone concentration</td>
<td>52</td>
</tr>
<tr>
<td>6.</td>
<td>[S]/v versus [S] plot for the 11β-hydroxylation of deoxycorticosterone</td>
<td>54</td>
</tr>
<tr>
<td>7.</td>
<td>Reduced minus oxidized difference spectrum of the mitochondrial acetone powder enzyme preparation reduced with NADPH or sodium dithionite</td>
<td>56</td>
</tr>
<tr>
<td>8.</td>
<td>Carbon monoxide difference spectrum of the mitochondrial acetone powder enzyme preparation reduced with NADPH or sodium dithionite</td>
<td>58</td>
</tr>
<tr>
<td>9a.</td>
<td>Dithionite reduced minus oxidized difference spectrum of the 0-20% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>60</td>
</tr>
<tr>
<td>9b.</td>
<td>Dithionite reduced carbon monoxide difference spectrum of the 0-20% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>60</td>
</tr>
<tr>
<td>10a.</td>
<td>Dithionite reduced minus oxidized difference spectrum of the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>61</td>
</tr>
<tr>
<td>10b.</td>
<td>Dithionite reduced carbon monoxide difference spectrum of the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>61</td>
</tr>
<tr>
<td>11a.</td>
<td>Dithionite reduced minus oxidized difference spectrum of the 40-80% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>62</td>
</tr>
<tr>
<td>11b.</td>
<td>Dithionite reduced carbon monoxide difference spectrum of the 40-80% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>62</td>
</tr>
<tr>
<td>12.</td>
<td>NADPH oxidation by an enzyme preparation obtained by ultracentrifugation of sonicated adrenal mitochondria</td>
<td>66</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>13.</td>
<td>NADPH reduced carbon monoxide difference spectrum of an enzyme preparation</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>obtained by ultracentrifugation of sonicated adrenal mitochondria</td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>Effect of extraction with acetone or acetone-water (9:1 v/v) on the</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>cytochrome P-450 content of the sonicated adrenal mitochondria enzyme</td>
<td></td>
</tr>
<tr>
<td></td>
<td>preparation</td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>Effect of incubation temperature on the recovery of cytochromes P-450 and</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>P-420 from the adrenal mitochondrial acetone powder enzyme preparation</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>Conversion of cytochrome P-450 to cytochrome P-420 as a function of</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>incubation time</td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td>Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>preparation after incubation in the presence of deoxycorticosterone or</td>
<td></td>
</tr>
<tr>
<td></td>
<td>corticosterone</td>
<td></td>
</tr>
<tr>
<td>18.</td>
<td>Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>preparation after incubation in the presence of androstenedione or adrenosterone</td>
<td></td>
</tr>
<tr>
<td>19.</td>
<td>Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>powder enzyme preparation after incubation in the presence of high</td>
<td></td>
</tr>
<tr>
<td></td>
<td>concentrations of deoxycorticosterone</td>
<td></td>
</tr>
<tr>
<td>20.</td>
<td>Effect of deoxycorticosterone on the rate of cytochrome P-450 disappearance</td>
<td>86</td>
</tr>
<tr>
<td>21.</td>
<td>Effect of the acetone-extracted mitochondrial lipid fraction on NADPH</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>oxidation</td>
<td></td>
</tr>
<tr>
<td>22.</td>
<td>Effect of menadione on NADPH oxidation</td>
<td>98</td>
</tr>
<tr>
<td>23.</td>
<td>Effect of 1,4-naphthoquinone, 1,4-toluquinone, and 1,4-benzoquinone on</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>NADPH oxidation</td>
<td></td>
</tr>
<tr>
<td>24.</td>
<td>Effect of deoxycorticosterone, mitochondrial acetone-extracted lipid, and</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>dicumarol on NADPH oxidation</td>
<td></td>
</tr>
<tr>
<td>25.</td>
<td>Effect of deoxycorticosterone, menadione, and dicumarol on NADPH oxidation</td>
<td>106</td>
</tr>
<tr>
<td>26.</td>
<td>Effect of mitochondrial acetone-extracted lipid, menadione, and dicumarol</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>on the 11β-hydroxylation of deoxycorticosterone</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>27a. Effect of menadione on the oxidation of NADPH by the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>27b. Effect of menadione on the NADPH mediated reduction of cytochromes P-450 and P-420 in the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>28. Effect of mitochondrial lipid and asolectin on the 11β-hydroxylation of deoxycorticosterone</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td>29. 11β-hydroxylation of deoxycorticosterone as a function of mitochondrial lipid or asolectin concentration</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>30. Effect of deoxycorticosterone and mitochondrial lipid on cytochrome P-450</td>
<td>127</td>
<td></td>
</tr>
<tr>
<td>31. Effect of total mitochondrial lipid and -20°C acetone-insoluble fraction on cytochromes P-450 and P-420</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>32. Recovery of cytochromes P-450 and P-420 as a function of the mitochondrial lipid concentration</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>33. Recovery of cytochromes P-450 and P-420 as a function of asolectin concentration</td>
<td>131</td>
<td></td>
</tr>
<tr>
<td>34. Effect of mitochondrial lipid on the rate of cytochrome P-450 disappearance</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td>35. Schematic representation of the interaction of menadione with the components of the electron transport pathway for 11β-hydroxylation</td>
<td>137</td>
<td></td>
</tr>
<tr>
<td>36a. Effect of dicumarol and deoxycorticosterone on NADPH oxidation</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>36b. Effect of dicumarol on the 11β-hydroxylation of deoxycorticosterone</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>37. Inhibition of the 11β-hydroxylation of deoxycorticosterone as a function of dicumarol concentration</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>38. [S]/v versus [S] plot of dicumarol inhibition of the 11β-hydroxylation of deoxycorticosterone</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>39. 1/v versus i plot of dicumarol inhibition of the 11β-hydroxylation of deoxycorticosterone</td>
<td>148</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>40.</td>
<td>NADPH oxidation as a function of Metopirone concentration</td>
<td></td>
</tr>
<tr>
<td>41.</td>
<td>Inhibition of the 11β-hydroxylation of deoxycorticosterone as a function of Metopirone concentration</td>
<td></td>
</tr>
<tr>
<td>42a.</td>
<td>Effect of Metopirone and deoxycorticosterone on NADPH oxidation</td>
<td></td>
</tr>
<tr>
<td>42b.</td>
<td>Effect of Metopirone on the 11β-hydroxylation of deoxycorticosterone</td>
<td></td>
</tr>
<tr>
<td>43.</td>
<td>1/v versus i plot of Metopirone inhibition of 11β-hydroxylation of deoxycorticosterone</td>
<td></td>
</tr>
<tr>
<td>44.</td>
<td>Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of dicumarol</td>
<td></td>
</tr>
<tr>
<td>45.</td>
<td>Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of low concentrations of dicumarol</td>
<td></td>
</tr>
<tr>
<td>46.</td>
<td>Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of high concentrations of dicumarol</td>
<td></td>
</tr>
<tr>
<td>47.</td>
<td>Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of dicumarol and deoxycorticosterone</td>
<td></td>
</tr>
<tr>
<td>48.</td>
<td>Effect of deoxycorticosterone and dicumarol on the rate of cytochrome P-450 disappearance</td>
<td></td>
</tr>
<tr>
<td>49.</td>
<td>Effect of dicumarol on cytochrome P-450 in the 11β-hydroxylase prepared from sonicated adrenal mitochondria</td>
<td></td>
</tr>
<tr>
<td>50.</td>
<td>Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of Metopirone</td>
<td></td>
</tr>
<tr>
<td>51.</td>
<td>Effect of Metopirone on the rate of cytochrome P-450 disappearance</td>
<td></td>
</tr>
<tr>
<td>52.</td>
<td>Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of low concentrations of Metopirone</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>53. Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of Metopirone and NADPH</td>
<td>186</td>
<td></td>
</tr>
<tr>
<td>54. Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of Metopirone and deoxycorticosterone</td>
<td>188</td>
<td></td>
</tr>
<tr>
<td>55. Spectral changes produced by addition of deoxycorticosterone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>192</td>
<td></td>
</tr>
<tr>
<td>56. The effect of deoxycorticosterone concentration on the magnitude of the spectral change produced by addition of deoxycorticosterone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>194</td>
<td></td>
</tr>
<tr>
<td>57. Double reciprocal plot of the change in absorbance (385-420 m(\mu)) or (420-403 m(\mu)) as a function of deoxycorticosterone concentration</td>
<td>195</td>
<td></td>
</tr>
<tr>
<td>58. Effect of dicumarol on the spectral changes produced by addition of deoxycorticosterone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>197</td>
<td></td>
</tr>
<tr>
<td>59. Effect of dicumarol on the magnitude of the spectral change (385-420 m(\mu)) produced by addition of deoxycorticosterone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>198</td>
<td></td>
</tr>
<tr>
<td>60. Spectral changes produced by addition of Metopirone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>201</td>
<td></td>
</tr>
<tr>
<td>61. Effect of Metopirone on the spectral changes produced by addition of deoxycorticosterone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>203</td>
<td></td>
</tr>
<tr>
<td>62. Effect of Metopirone on the magnitude of the spectral change (385-420 m(\mu)) produced by addition of deoxycorticosterone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation</td>
<td>204</td>
<td></td>
</tr>
<tr>
<td>63. Schematic representation of the conversion of cytochrome P-450 to different conformational states by interaction with deoxycorticosterone, Metopirone, and dicumarol</td>
<td>217</td>
<td></td>
</tr>
</tbody>
</table>
PUBLICATIONS


ABBREVIATIONS USED

Deoxycorticosterone: 21-hydroxypregn-4-ene-3,20-dione
Corticosterone: 11β,21-dihydroxypregn-4-ene-3,20-dione
Deoxycortisol: 17,21-dihydroxypregn-4-ene-3,20-dione
Androstenedione: androst-4-ene-3,17-dione
Testosterone: 17β-hydroxyandrost-4-en-3-one
11β-Hydroxyandrostenedione: 11β-hydroxyandrost-4-ene-3,17-dione
Adrenosterone: androst-4-ene-3,11,17-trione
Dehydroepiandrosterone: 3β-hydroxyandrost-5-en-17-one
Dicumarol: 3,3'-methylenebis-(4-hydroxycoumarin)
Metopirone: 2-methyl-1,2-bis(3-pyridyl)-1-propanone
Menadione: 2-methyl-1,4-naphthoquinone
Coenzyme Q: 2,3-dimethoxy-5-methyl-6-multiprenyl-1,4-benzoquinone
NAD: oxidized nicotinamide-adenine dinucleotide
NADH: reduced NAD
NADP: oxidized nicotinamide-adenine dinucleotide phosphate
NADPH: reduced NADP
ATP: adenosine triphosphate
PPO: 2,5-diphenyloxazole
POPOP: 1,4-bis[2-(5-phenyloxazolyl)]benzene
ACKNOWLEDGEMENTS

I wish to express my deepest thanks to Dr. V.J. O'Donnell for his guidance and encouragement throughout these studies and for his invaluable assistance in the preparation of this thesis. I would also like to thank Dr. P.D. Bragg for his advice on the experiments with lipoquinones and on the technique of difference spectrophotometry.

I would like to acknowledge the National Research Council of Canada and the H.R. MacMillan Family for financial support in the form of scholarships awarded to me during these studies. The research was supported by grants to Dr. V.J. O'Donnell from the Medical Research Council of Canada and from the National Cancer Institute of Canada.
INTRODUCTION

In 1955 Mason, Fowlks, and Peterson observed (1) that during the enzymatic oxidation of 3,4-dimethylphenol to 4,5-dimethylcatechol the oxygen atom incorporated into the substrate molecule was derived exclusively from molecular oxygen. This observation has led to the discovery of a number of enzymes catalyzing similar reactions (2,3). Hayaishi (2) introduced the term "oxygenase" to describe enzymes of this type. These enzymes in general catalyze the introduction of one or two atoms of molecular oxygen into the substrate molecule. In oxidation reactions in which only one atom of molecular oxygen is incorporated into the substrate, the second atom is reduced to water in the presence of an appropriate electron donor such as NADH, NADPH, tetrahydrofolate, or ascorbic acid (2-4). Mason (3) introduced the term "mixed function oxidase" to describe enzymes catalyzing this particular type of reaction because they possess both oxygenase activity (incorporation of an atom of molecular oxygen into the substrate), and oxidase activity (reduction of an atom of molecular oxygen to water). The terms "monooxygenase" (5) and "hydroxylase" (6) have also been employed to describe these enzymes. The properties and requirements of the enzymes catalyzing hydroxylations of steroid hormones place them in the group of mixed function oxidases.

The requirements of the mixed function oxidases are unusual in that oxygen is required as a specific oxidizing agent
and an electron donor is needed as a reductant. In steroid hydroxylases NADPH is usually the reductant (7,8,9) while some other types of hydroxylases are specific for NADH (10, 11). Ascorbic acid serves as reductant in dopamine hydroxylase (12) and tetrahydropteridine is required in the phenylalanine hydroxylase system (13).

Steroid Hydroxylases

The steroid molecule is hydroxylated at a number of positions in both animal tissues (14) and bacteria (15-18). Major efforts in the study of the enzymatic mechanism of mammalian steroid hydroxylations have been directed toward the 11β-, 17α-, and 21-hydroxylases of the adrenal cortex (14,19). Of these, the 11β-hydroxylase has been studied most extensively and is the subject of this thesis. The 11β-hydroxyl function of corticosteroids has had particular significance attached to it in terms of hormone activity by Bush and Mahesh (20). They suggest that the biological activity of 11-oxygenated steroids as glucocorticoids may be due to the specific interaction of an 11β-hydroxyl group with the receptor site for these hormones.

Steroid 11β-Hydroxylase

The steroid 11β-hydroxylase is located in the mitochondrial fraction of adrenocortical tissue (21-25). Also present in this subcellular fraction are the 18-hydroxylase (26) and hydroxylases involved in the side-chain cleavage of cholesterol to pregnenolone (27). The 17α- and 21-hydroxylases on the
other hand are associated with the endoplasmic reticulum (28-30).

The 11β-hydroxylase of the adrenal cortex has been prepared by a variety of methods. The system can be extracted from an acetone powder of adrenal mitochondria (31) and partially extracted from lyophilized mitochondria (32). Sharma et al. (33) have obtained an active preparation from bovine adrenal cortex by ultrasonic treatment of adrenal mitochondria followed by centrifugation. The properties of the 11β-hydroxylase from these various preparations have been examined in detail.

In early studies on the cofactor requirements of the 11β-hydroxylase a number of components appeared essential. An oxygen-containing atmosphere was required for hydroxylation (22,24,25,34). The reaction also appeared to be dependent on, or stimulated by a variety of factors, including ATP, Mg++, NAD, NADP, nicotinamide and fumarate or other Krebs cycle intermediates (22-25). In addition, Brownie and Grant (24) noted that succinate-supported 11β-hydroxylation was inhibited by dinitrophenyl, suggesting that oxidative phosphorylation was necessary for 11β-hydroxylation. The function of the various cofactors was clarified by Grant (31) and by Sweat and Lipscomb (8) who independently showed that NADPH was the factor involved in steroid 11β-hydroxylation and that all the other compounds were associated with the intramitochondrial production of the reduced coenzyme.
In the enzymatic 11β-hydroxylation reaction the hydroxyl group introduced directly replaces the hydrogen atom of the same steric configuration without any inversion occurring (35, 36). Studies on the mechanism of hydroxylation by Hayano and Dorfman (23) indicate that the \( \Delta^9(11) \) or the \( \Delta^{11}(12) \) unsaturated analogues of deoxycorticosterone are not intermediates of 11β-hydroxylation. Incubations carried out in \( \text{H}_2\text{O}^{18} \) result in no incorporation of \( O^{18} \) into the 11β-hydroxylated product (9). Thus 11β-hydroxylation does not involve a dehydrogenation reaction followed by subsequent hydration. Hayano et al. (9) have demonstrated, using \( O_2^{18} \), that the oxygen of the 11β-hydroxyl function is derived totally from molecular oxygen. The 11β-hydroxyl group is stable and no exchange is observed when incubations are carried out in the presence of \( \text{D}_2\text{O} \) (23) or \( \text{H}_2\text{O}^{18} \) (9). The direct involvement of molecular oxygen in 11β-hydroxylation has also been reported by Sweat et al. (37). The demonstration of incorporation of molecular oxygen into the steroid molecule explains the earlier observations that this reaction proceeded only in an oxygen atmosphere (22,24, 25,34). 11β-hydroxylation does not proceed via the formation of an epoxide intermediate. The formation of small amounts of the \( 9\beta,11\beta \)-epoxide from \( \Delta^9(11) \)-11-deoxycortisol has been observed (38) but this compound is not further metabolized.

The stoichiometric relationship between steroid substrate, molecular oxygen, and NADPH has not been firmly established for 11β-hydroxylation. Recently Cammer and Estabrook (39) have
shown that the molar ratio of O₂ uptake and 11β-hydroxylation in beef adrenal mitochondria is unity. Cooper et al. (40) have established the stoichiometry for the 21-hydroxylase of adrenal microsomes. One molecule of NADPH is oxidized for each atom of molecular oxygen incorporated into the C-21 position of the steroid substrate.

While the cofactor requirements and properties of the 11β-hydroxylase are quite well established, very little is known about this system in terms of substrate specificity. Sharma et al. (33,41) have shown that the 11β-hydroxylation of deoxycorticosterone is inhibited competitively by 11-deoxycorticisol, androstenedione, testosterone, and dehydroepiandrosterone; whereas 11β-hydroxyandrostenedione is not inhibitory. It is likely that the inhibitory effect of these steroids is due to their participation as substrates in the hydroxylation reaction. Several drugs are also known to inhibit 11β-hydroxylation (41-43). Metopirone [2-methyl-1,2-bis(3-pyridyl)-1-propanone] inhibition of 11β-hydroxylation has been studied extensively (42-49). This compound appears to be relatively specific for mitochondrial steroid hydroxylases, e.g., 11β- and 18-hydroxylation.

Detailed studies on the interaction of steroid substrates with the 11β-hydroxylase and on the mode of action of inhibitors of the hydroxylation reaction may provide much information on the overall mechanism of this mixed function oxidase. The mechanisms of action of two 11β-hydroxylase inhibitors, metopirone and dicumarol, have been examined in this laboratory.
and the results obtained are presented in this thesis.

**Components of the 11β-Hydroxylase**

The first indication of the complex nature of the 11β-hydroxylase was provided by Tomkins *et al.* (50,51) who extracted three heat-labile enzyme fractions from an adrenal mitochondrial acetone powder, all of which were required to reconstitute 11β-hydroxylase activity. The activity could be stimulated by addition of a boiled extract of an acetone powder of adrenal or liver tissue. This heat-stable factor was destroyed on ashing, was not adsorbed on charcoal at an acid pH, and was neither tetrahydrofolate, \( \text{H}_2\text{O}_2 \), ascorbate nor dihydroxyfumarate. Sweat and Bryson (52) were able to separate the 11β-hydroxylase into two components by ammonium sulfate fractionation. Nakamura and associates (53,54) demonstrated that the heat-stable fraction first reported by Tomkins was protein in nature and stimulated 11β- and 18-hydroxylation, but not 21-hydroxylation. This heat-stable protein has been identified as a nonheme iron protein, and has been named adrenodoxin (55).

Omura *et al.* (56,57) isolated and partially purified three fractions from beef adrenal mitochondria that when combined could reconstitute 11β-hydroxylase activity. These three fractions consisted of a NADPH flavoprotein reductase, a nonheme iron protein, and an unusual hemoprotein called cytochrome P-450. They postulated the pathway for hydroxylation illustrated in Figure 1. The isolation and identification of similar components of the rat and porcine adrenal
Fig. 1. Scheme proposed by Omura et al. (56) for the electron transport pathway in hydroxylation reactions.

* Indicates reduced forms of Fp and P₄₅₀.
11β- and 18-hydroxylase were also reported by Nakamura and Otsuka (32). The established components of the 11β-hydroxylase will be discussed individually.

NADPH Reductase

The flavoprotein that catalyzes the oxidation of NADPH in adrenal mitochondria has been called NADPH-diaporase (57) and adrenodoxin reductase (55,58,59). This enzyme catalyzes the transfer of electrons from NADPH to the nonheme iron component of the 11β-hydroxylase pathway. In addition to the natural nonheme iron protein, this flavoprotein may also utilize ferri-cyanide, menadione, and cytochrome c as electron acceptors (57, 60). NADH is not oxidized by the purified enzyme (57). This NADPH reductase is similar to the NADPH-cytochrome c reductase of liver microsomes (61,62) in terms of the specific compounds it may utilize as electron acceptors.

Nonheme Iron Protein

The natural electron acceptor of the adrenal mitochondrial NADPH reductase is a nonheme iron protein that has been named adrenodoxin (55). This protein has been purified extensively by Kimura and Suzuki (59) and has been shown to be required in 11β-hydroxylation (55,58,60). The purified protein contains two iron atoms and two moles of acid-labile sulfide per mole of protein and has a molecular weight of 15,000-20,000. Combination of adrenodoxin and NADPH reductase reconstitutes the NADPH-cytochrome P-450 reductase activity of adrenal mitochondria (57); thus adrenodoxin is placed between the flavoprotein
and cytochrome P-450 in the electron transport sequence proposed by Omura et al. (Fig. 1). Although microsomal hydroxylations are believed to proceed in a manner analogous to those of the mitochondria, nonheme iron protein similar to adrenodoxin has not been demonstrated in this fraction (63). Mitochondrial adrenodoxin is similar in its physicochemical properties to ferredoxin (64,65) and to photosynthetic pyridine nucleotide reductase (66) but the latter protein cannot substitute for adrenodoxin in 11β-hydroxylation (58).

**Cytochrome P-450**

Cytochrome P-450 is an unusual hemoprotein that in the reduced form is capable of combining with carbon monoxide to form a complex exhibiting an absorption maximum at 450 m\(\mu\) (56, 67). The first implication for a role of this cytochrome in steroid hydroxylations was provided by Ryan and Engel (30) who observed that the 21-hydroxylase of beef adrenal microsomes was inhibited by carbon monoxide. However they did not study the nature of the carbon monoxide inhibition. Estabrook et al. (68) demonstrated during studies on the light-reversibility of the carbon monoxide inhibition of steroid 21-hydroxylation that maximal reversibility of inhibition was achieved at 450 m\(\mu\), corresponding to the absorption maximum of the cytochrome P-450-carbon monoxide complex. The presence of a similar cytochrome was observed in rat and bovine adrenal mitochondria (67-71) and Wilson et al. (72) demonstrated that this hemo-
protein was involved in steroid 11β-hydroxylation. Cytochrome P-450 has since been observed in a number of tissues, including liver, kidney, small intestine, adrenal cortex and corpus luteum (73-75). Cytochrome P-450 has been shown to participate in a number of mixed function oxidase reactions, including the 11β-, 18-, and 21-hydroxylation of steroids (68,72,76,77), the conversion of cholesterol to pregnenolone (78-80), microsomal drug hydroxylation and demethylation (81,82), and lipid metabolism (83). It is believed to be the terminal oxidase of mixed function oxidase systems.

All attempts to purify cytochrome P-450 have been unsuccessful because of the extreme instability of this hemoprotein. Therefore the properties of this hemoprotein have been studied mainly by the technique of difference spectrophotometry. The reduced minus oxidized difference spectrum of cytochrome P-450 has been difficult to determine because of the coexistence in microsomes and mitochondria of other redox components. Nishibayashi and Sato (84) have reported that cytochrome P-450 of rabbit liver microsomes exhibits a difference spectrum with two broad absorption maxima at 445 and 555 μ. These absorption maxima are of low intensity. Cammer and Estabrook (85) observed that the difference spectrum of cytochrome P-450 of beef adrenal mitochondria has two absorption maxima at 432 and 555 μ. Upon combination of the reduced form of cytochrome P-450 with carbon monoxide an intense absorption maximum at 450 μ is observed (56,67,69). This absorption maximum is unique in that it occurs at
a wavelength higher than that normally ascribed to carbon monoxide complexes of hemoproteins (56).

Cytochrome P-450 may be isolated by sonication of mitochondria (56,86) or by solubilization of microsomes with non-ionic detergents (87). Cytochrome P-450 prepared by sonication of mitochondria is particulate in nature and can be sedimented by ultracentrifugation (56). The unique spectral properties of cytochrome P-450 appear to be maintained by the structural association of this hemoprotein with membranes. Omura and Sato (88,89) observed that treatment of a rabbit liver microsomal preparation with a 0.1% solution of sodium deoxycholate or a heated snake venom preparation resulted in a loss of the absorption maximum at 450 µm in the carbon monoxide difference spectrum of the microsomes with the formation of a new absorption maximum at 420 µm. This new maximum was due to a hemoprotein that they termed cytochrome P-420. They concluded that solubilization of the microsomes resulted in the conversion of cytochrome P-450 to P-420. A large number of agents are now known that elicit the conversion of cytochrome P-450 to P-420. These include sodium deoxycholate (88,89), snake venom (88,89), lyssolecithin (90), trypsin (81), urea (81), bathocuproine sulfonate (91), guanidine hydrochloride (90), neutral salts (90), parachloromercuribenzoate (92), and organic solvents (81,90,93).

Cytochrome P-450 is closely associated with phospholipid. The specific structural relationship of the heme to the hydrophobic regions of the hemoprotein is maintained by the con-
formation of the cytochrome P-450 protein and by the phospholipid in the environment. Ichikawa and Yamano (93) maintain that the hydrophobic bonding in cytochrome P-450 is important in that it binds the heme to the apoprotein. The conversion of cytochrome P-450 to cytochrome P-420 results from a disturbance of the hydrophobic environment around the heme, either through a conformational change in the hemoprotein or by the removal of a hydrophobic moiety such as phospholipid. Mason et al. (81) have proposed a structure for microsomal cytochrome P-450 based on the effects of various agents on the hemoprotein. They suggest that in mixed-function oxidation, provision must be made for both oxygen activation and electron transport at the active site, and that a conformational change must occur during catalysis to allow the substrate, oxygen, and two reducing equivalents to interact. Further, these authors propose that cytochrome P-450 is a phospholipid complex of the sulfide of cytochrome P-420; that is, that the phospholipid moiety is linked to the hemoprotein, cytochrome P-420, through a sulfide bridge. The phospholipid component affects the presentation of the substrate to the oxidase. Substrate, oxygen, and sulfide interact at the protoheme group, the sulfide serving as the terminus of an electron transport chain that supplies the required two reducing equivalents to the mixed-function oxidase.
Cytochrome P-420

Cytochrome P-420, obtained by treatment of a rabbit liver microsomal fraction with heated snake venom, has been purified extensively by Omura and Sato (89). They observed that the reduced minus oxidized difference spectrum of the soluble hemoprotein was very similar to that of cytochrome $b_1$ of bacteria. Unlike the $b$ cytochromes however, cytochrome P-420, in its reduced state, combines with carbon monoxide, forming a complex exhibiting an absorption maximum at 420 m$\mu$. Although the cytochrome P-420 purified by Omura and Sato (89) is soluble, particulate forms also exist (90). Mason et al. (81) have presented evidence for the existence of several states of cytochrome P-420. These forms all exhibit the same absorption spectra but differ from one another in their spin states as determined by electron paramagnetic resonance spectroscopy. Ichikawa and Yamano (94) have observed that detergent and sulfhydryl reagent-produced cytochrome P-420 can be reconverted to cytochrome P-450 by the addition of polyols or glutathione. Such reconversion could not be effected on cytochrome P-420 produced by treatment with phospholipase, urea, or guanidine salts. They have proposed a scheme for the formation of various states of cytochrome P-420 dependent upon the extent of degradation of cytochrome P-450 (93,94). Removal of phospholipid or protein from cytochrome P-450 yields a form of cytochrome P-420 which cannot revert to cytochrome P-450. Thus procedures that tend only to interfere with the hydro-
phobic bonding in cytochrome P-450 produce a state of cytochrome P-420 than can be reconverted to cytochrome P-450.

Cytochrome P-420 can be enzymatically reduced with NADPH (88,89) but the purified cytochrome does not interact directly with the coenzyme. The hemoprotein can also accept electrons from adrenodoxin (59,60). However it does not function in hydroxylation reactions. The hydrophobic alteration accompanying the conversion of cytochrome P-450 to cytochrome P-420 results in a concomitant loss of its ability to interact with substrates for hydroxylation.

**Cytochrome P-450 as the Substrate Binding Site**

Spectrophotometric evidence indicates that cytochrome P-450 is the substrate-binding site of mixed function oxidases (95-98). Addition of the appropriate substrate to mitochondrial or microsomal hydroxylase systems produces spectral changes that can be measured by difference spectrophotometry. Imai and Sato (98) have presented evidence that the substrate-produced spectral changes involve cytochrome P-450 and that the interaction of substrate with the hemoprotein is an obligatory step in the mechanism of hydroxylation. Cammer and Estabrook (85) have noted that deoxycorticosterone induces spectral changes in enzymatically reduced cytochrome P-450 of adrenal mitochondria that differ from those produced in oxidized cytochrome P-450. They have outlined a sequence of oxidation and reduction changes in cytochrome P-450 that occur during the course of steroid 11β-hydroxylation.
The spectral changes in cytochrome P-450 produced by substrates are believed to result from the interaction of the substrate with the protein moiety of the hemoprotein. This interaction produces conformational changes in the cytochrome that cause an alteration in the interaction of ligands with the heme. This hypothesis is supported by evidence obtained by electron paramagnetic resonance spectroscopy that indicates that substrate binding modifies ligand interaction with the heme iron of cytochrome P-450 (99, 100). The spectral changes can be induced by a number of substrates of the mitochondrial and microsomal hydroxylases as well as by some inhibitors of these hydroxylases and by organic solvents (97,98). The type of spectral change appears to vary with the compound employed. Schenkman et al. (97) have divided substrates and inhibitors of the microsomal hydroxylase into two categories depending on the type of spectral change produced. That different spectral changes are elicited by different substrates implies that cytochrome P-450 possesses more than one binding site for such compounds.

The observations on the binding of substrates to cytochrome P-450 suggest that the hemoprotein may exist in more than one state, and that conversion from one state to another results from a conformational transition. Supporting evidence for the existence of more than one form of cytochrome P-450 has been provided by Sato and associates (101-104). Combination of ethyl isocyanide with the reduced form of microsomal cytochrome P-450 produces a complex exhibiting
an absorption spectrum with maxima at 430 m\( \mu \) and 455 m\( \mu \). The intensity of these absorption maxima are influenced by pH in an interdependent manner. Similar, but much weaker absorption maxima are produced by aniline, a substrate of the microsomal hydroxylase (104). Imai and Sato (104) have concluded that reduced cytochrome P-450, in combination with lipophilic ligands such as ethyl isocyanide or aniline, exists in two interconvertible states that are in a pH-dependent equilibrium.

While cytochrome P-450 appears to be the site for both substrate and oxygen activation in mixed function oxidation, very little is known concerning the interaction of oxygen with substrate. On the basis of studies with model chemical systems, Ullrich and Staudinger (105) suggest that a form of the oxygen atom itself is the hydroxylating particle in mixed function oxidases. Oxygen is first attached to the divalent iron of reduced cytochrome P-450 (67) which then is oxidized immediately. The complex formed is a stabilized form of \( O_2^- \) (or \( O_2H \)) which is then reduced by a second electron from NADPH. An iron-oxene complex is formed with the same oxidizing properties as an oxygen atom, but with one of the two unpaired electrons attached to ferric iron.

While many of the components of the mitochondrial and microsomal hydroxylase systems have been identified, little is known concerning the substrate specificity of these systems. Liver microsomal hydroxylase preparations are capable of hydroxylating a wide variety of structurally unrelated substrates. Both 17\( \alpha \)- and 21-hydroxylases are present in the
endoplasmic reticulum of adrenocortical tissue. In adrenocortical mitochondria at least three steroid reactions are known to involve a cytochrome P-450, i.e., 11β-, 18-hydroxylation, and cholesterol sidechain cleavage. Whether a single cytochrome P-450, and its associated electron transport chain, functions in all three reactions is not known. Nakamura and Otsuka(32) separated a protein component of rat adrenal mitochondria that they reported determined the carbon atom on the steroid to be hydroxylated; that is, 11β- or 18. Further studies on this component have not been reported, and the question whether additional enzymes or factors exist that confer specificity to the hydroxylase reaction must await further investigations.

This thesis reports on the mechanisms of action of two inhibitors of 11β-hydroxylation, namely, dicumarol and Metopirone. The interactions of the inhibitors and substrate deoxycorticosterone with the 11β-hydroxylase pathway have been examined. The 11β-hydroxylation reaction studied is outlined in Figure 2. The structural formulae of the inhibitors dicumarol and Metopirone are illustrated in Figure 3. Elucidation of the mechanisms of inhibition by these compounds may lead to a further understanding of the specificity of the 11β-hydroxylase, the interaction of steroid substrate with this system, and the structural features of both steroid substrates and inhibitors that allow them to interact with the 11β-hydroxylase of adrenocortical mitochondria.
Fig. 2. The transformation of deoxycorticosterone to corticosterone.

Fig. 3. Structural formulae of dicumarol and Metopirone.
MATERIALS AND METHODS

(a) Solvents

Solvents (AR grade) were purified by distillation. Acetone was refluxed for one hour with potassium permanganate and potassium carbonate and then distilled twice. Ethanol was refluxed for two hours with potassium hydroxide and zinc and then distilled twice. Ether was washed with a dilute, weakly acidic solution of ferrous sulfate (3 x 1/10 vol), then to neutrality with distilled water, and distilled from calcium hydride under anhydrous conditions. Spectroscopic grade isooctane (British Drug Houses, Ltd.) was employed without purification.

(b) Chemicals

Tris(hydroxymethyl)aminomethane (Trizma base, reagent grade) was purchased from Sigma Chemical Company. Tris buffer was prepared by titrating a solution of this compound to the desired pH with hydrochloric acid. NADPH (A grade) was purchased from Calbiochem, Inc. Dicumarol [3,3'-Methylenebis-(4-hydroxycoumarin)] was a gift of the Wisconsin Agricultural Research Foundation and Abbott Laboratories. A solution (0.01 M) of dicumarol was prepared by suspending 25 mg of the compound in 2.5 ml of 75% ethanol. A solution of 1 N potassium hydroxide was added dropwise (4 or 5 drops) to the continually warmed suspension until all the dicumarol dissolved. The solution was then diluted to 7.5 ml with distilled water. Metopirone [2-methyl-1,2-bis(3-pyridyl)-1-propanone] was a gift of Ciba Company Limited. This compound was employed dissolved in a 0.1 M solution of Tris buffer containing 2.5 x
$10^{-3}$ M MgCl$_2$.

(c) Steroids

Deoxycorticosterone, purchased from Sigma Chemical Company, was purified by crystallizations from acetone-hexane. Deoxycorticosterone-1,2-$^3$H (Tracerlab) and deoxycorticosterone-4-$^{14}$C (New England Nuclear Corporation) were purified by paper-partition chromatography in the solvent system ligroin-propylene glycol (106) and diluted with radioinert deoxycorticosterone to the final specific activities indicated in the text.

Radioinert corticosterone, purchased from Mann Research Laboratories, Inc., was purified first by thin-layer chromatography on silica gel G containing Radelin GS-115 phosphor in an ethyl acetate solvent system. Two ultra-violet absorbing zones were obtained, one corresponding in mobility to corticosterone, and a second less polar zone present in a minor amount. The corticosterone zone was scraped off, eluted with methanol, and twice crystallized from acetone-hexane.

(d) Chromatography Procedures

Silica gel G (according to Stahl) containing Radelin GS-115 phosphor was employed for thin-layer chromatography. Silica gel G was washed with methanol and then dried. Radelin GS-115 phosphor was extracted with methanol in a soxhlet and then dried. To prepare the chromatography plates, 50 g of silica gel G, 25 mg of Radelin GS-115 phosphor, and 100 ml of distilled water were mixed thoroughly. The slurry was then spread on glass plates (20 cm x 20 cm or 10 cm x 20 cm) with
a Desaga apparatus. The plates were then dried in an oven (~80° C) and stored in a desiccator cabinet.

Paper-partition chromatography was carried out with Whatman No. 1 chromatogram paper that had been previously washed with methanol to remove ultra-violet absorbing material. The chromatogram paper was cut into strips, usually 2 cm in width, for use in the paper-partition chromatography systems.

(e) Isolation of the 11β-Hydroxylase from Beef Adrenal Glands

(i) Preparation of beef adrenal mitochondria:

Beef adrenal glands obtained from the slaughterhouse were freed of adhering fat and connective tissue, dipped into a 0.25 M sucrose solution at 4° C, and stored at -20° C until they were processed. All steps in the preparation of the adrenal mitochondria were carried out in the cold room (4° C). The beef adrenal glands, still partially frozen, were disrupted in a 0.25 M sucrose solution (w/v, 1:5) with a Waring blender and then homogenized with a Potter-Elvehjem type homogenizer. The homogenate was centrifuged (1,400 x g for 15 minutes) and the supernatant was filtered through gauze and centrifuged (10,000 x g for 25 minutes) to yield the mitochondrial pellet. The pellet was washed twice by resuspension in three volumes of 0.25 M sucrose solution followed each time by centrifugation at 12,000 x g for 30 minutes.

(ii) Preparation of the mitochondrial acetone powder:

The washed mitochondrial pellet was suspended in a minimal volume of a 0.25 M sucrose solution and the suspension was
poured, with stirring, into ten volumes of acetone (-20° C). The mixture, after standing at -20° C for five minutes, was centrifuged and the precipitate was extracted with three volumes of acetone (-20° C). After three such extractions the precipitate was dried in vacuo and stored over anhydrous calcium sulfate in a desiccator at -10° C. The mitochondrial acetone powder could be stored for at least three months without loss of 11β-hydroxylase activity. Approximately 1 g of acetone powder was obtained from 100 g of beef adrenal glands.

The 11β-hydroxylase system was extracted from the mitochondrial acetone powder, immediately before use in incubations, by the method outlined by Grant (31). The mitochondrial acetone powder was homogenized in an ice-cold solution of 0.154 M KCl, the homogenate was allowed to stand at 4° C for 30 minutes and then centrifuged at 17,000 x g for 30 minutes at 4° C. In all instances except where otherwise indicated, 1 g of acetone powder was extracted with 30 ml of a 0.154 M solution of KCl. In some preliminary experiments 1 g of acetone powder was extracted with 10 or 15 ml of a 0.154 M solution of KCl.

(iii) Preparation of the 11β-hydroxylase from sonicated adrenal mitochondria:

This 11β-hydroxylase system was prepared according to the method outlined by Sharma et al. (33). The washed mitochondrial pellet was homogenized with four times its weight of glass distilled water at 4° C. The homogenate was then sonicated for 15 minutes at 4° C in a Bronwill 20-kc sonic oscillator. Sonication was interrupted briefly after 5 and 10 minutes to
allow the apparatus and sonicate to cool. The sonicate was then centrifuged at 105,000 x g for 60 minutes. The resulting supernatant fluid was dried by lyophilization and the powder obtained was stored over anhydrous calcium sulfate in a desiccator at -10°C. The yield of lyophilized powder from 100 g of adrenal glands was 1.3 g. In incubations with this 11β-hydroxylase preparation, the desired amount of the lyophilized powder was weighed out and added directly to the incubation buffer mixture.

(f) **Protein Determinations**

The amount of protein present in the enzyme preparation extracted from the mitochondrial acetone powder and in the enzyme preparation from sonicated mitochondria was determined by the biuret method (107). The protein concentrations in the enzyme preparations were estimated by comparison to a standard solution of bovine serum albumin. The enzyme preparation extracted from 1 mg of mitochondrial acetone powder contains 0.225 mg of protein. One mg of lyophilized powder from sonicated mitochondria contains 0.240 mg of protein.

(g) **General Incubation Conditions**

In general, reactions were carried out in 25 ml Erlenmeyer flasks. A 0.1 M Tris solution containing 2.5 x 10⁻³ M MgCl₂ was employed as the buffer medium. The pH of the buffer was 7.4. Unless stated otherwise, all reactions were carried out at 37°C in a Dubnoff metabolic shaking incubator. The gas phase was air.
The order of addition of reaction components was generally as follows: steroid substrate, Tris buffer, enzyme preparation, experimental factors, NADPH. Deoxycorticosterone substrate was added dissolved in propylene glycol. Alternatively the substrate was added to the incubation flask in methanol, the methanol was removed under nitrogen, and propylene glycol was then added to dissolve the steroid. Other factors or compounds to be tested were dissolved or suspended in the appropriate solvents and added to the reaction flask. Prior to the addition of NADPH, each reaction mixture was pre-incubated at 37°C for either 5 or 8 minutes with shaking. NADPH was added in Tris buffer medium at zero time.

Three general reactions or properties of the 11β-hydroxylase preparations were examined with regard to the effects of steroid substrates, inhibitors (dicumarol and Metopirone), and other factors or compounds. These reactions (properties) were: (1) NADPH oxidation, (2) 11β-hydroxylation, (3) reduction and stabilization of cytochromes P-450 and P-420. When examining the effects of the various factors or compounds on these reactions, control incubations (with the factor absent) were simultaneously carried out. The control reaction flask received the same amount of the solvent in which the test factor or compound (dissolved or suspended) was added to the experimental reaction flask.

(h) Studies on NADPH Oxidation

NADPH oxidation was measured in a Beckman DU Spectrophotometer by the diminution of absorbance at 340 μm against
a blank containing all incubation components except the reduced coenzyme. A typical reaction mixture for measurement of NADPH oxidation contained: propylene glycol (with or without deoxycorticosterone), 0.05 ml; Tris-MgCl₂ buffer, 0.90 ml; enzyme preparation, 2.0 ml; test factor or compound, 0.05-0.2 ml. These reaction components were pre-incubated for 5 minutes and NADPH (\( \sim 1 \mu \text{mole} \)) in 0.1 ml Tris buffer medium was added at zero time to give a final volume of usually 3.2 ml. The absorbance of the reaction mixture was then measured at 340 \( \mu \text{m} \) at time intervals, usually 5 minutes, starting at zero time.

(i) **Studies on Corticosterone Formation (11\( \beta \)-Hydroxylation)**

Corticosterone formation was assayed by the isolation and subsequent measurement of this compound after incubation of the hydroxylase system with substrate deoxycorticosterone. When deoxycorticosterone-1,2-\(^{3}\)H or deoxycorticosterone-4-\(^{14}\)C was employed as the substrate, the isolated corticosterone was assayed for radioactivity. In preliminary experiments non-radioactive deoxycorticosterone was employed.

The concentrations of reaction components, incubation volumes, and incubation times varied slightly with each experiment. Reaction mixtures were generally set up as for the studies on NADPH oxidation. Reaction mixtures were pre-incubated for either 5 or 8 minutes and NADPH was added at zero time. Studies with radioactive deoxycorticosterone showed that essentially no conversion of this substrate to
corticosterone took place during this pre-incubation period in the absence of NADPH; the amount of radioactive corticosterone isolated after pre-incubation was 0.5% of that isolated after incubation in the presence of NADPH. After the desired incubation period, reactions were terminated by the addition of either ice-cold chloroform or ethyl acetate (5 or 15 ml).

(i) Kinetic studies on 11β-hydroxylation:
A typical incubation mixture was set up to contain: deoxycorticosterone-1,2-3H dissolved in 0.05 ml propylene glycol; Tris-MgCl₂ buffer, 0.70 ml; enzyme preparation, 1.3 ml; test factor or compound dissolved in 0.05 ml of the appropriate solvent. The mixture was kept in an ice-water bath throughout these additions. Each component was added at identical time intervals to each flask. Each reaction mixture was then pre-incubated with shaking at 37°C for 8 minutes. NADPH was added in 0.05 ml Tris-MgCl₂ buffer at zero time. After 90 seconds of incubation, a 1.0 ml sample was removed from each flask, pipetted into 2 ml of ice-cold ethyl acetate, and mixed thoroughly with a Vortex-Genie mixer.

(ii) Isolation, purification, and measurement of deoxycorticosterone and corticosterone:
In some instances, the chloroform- or ethyl acetate-arrested reaction mixtures were transferred to separatory funnels for extraction. Each mixture was diluted with water to 15 ml and then extracted four times with chloroform or ethyl acetate (15 ml). The combined solvent fraction was
evaporated to dryness under reduced pressure in a warm water bath and the residue redissolved in a minimal amount of methanol prior to separation of the steroids. In the kinetic experiments, and in the time studies carried out when 1 ml samples had been removed at time intervals from each reaction mixture, extractions were carried out in 12 ml centrifuge tubes. The reaction sample was extracted four times with chloroform or ethyl acetate (2 ml), and the solvent removed under nitrogen. The radioactivity recovered by both extraction procedures accounted for at least 95% of the added substrate radioactivity.

In preliminary experiments on the NADPH requirement of the 11β-hydroxylase and the effects of electron transport inhibitors on 11β-hydroxylation, deoxycorticosterone and corticosterone were isolated from the organic solvent extracts by thin-layer chromatography on silica gel G. The material remaining after removal of the organic solvent used in extraction was dissolved in a minimal amount of methanol and spotted on the chromatography plate. After development of the chromatogram in ethyl acetate, ultra-violet absorbing zones corresponding in mobility to authentic samples of deoxycorticosterone and corticosterone were scraped off and eluted with methanol. The quantity of eluted steroid was measured spectrophotometrically by its absorption at or about 240 μμ in methanol solution.

When deoxycorticosterone-1,2-3H or deoxycorticosterone-4-14C was employed as the substrate, 50 or 100 μg of radioinert
carrier corticosterone were added to the organic solvent extract material and the mixture was subjected to paper-partition chromatography in the solvent system toluene-propylene glycol (108) for 18 hours. Under these conditions substrate deoxycorticosterone was recovered in the solvent overflow. The corticosterone zones were located by ultra-violet absorption, excised, and eluted with methanol. The corticosterone eluates were assayed for radioactivity and the quantity of eluted steroid was measured spectrophotometrically at 241 μ in methanol solution.

Chromatography in the solvent system toluene-propylene glycol under these conditions, resolves the two steroids corticosterone and 18-hydroxy-deoxycorticosterone. Formation of the latter steroid has been observed in incubations with adrenal mitochondria (26,49). In some of the present experiments, incubation of deoxycorticosterone-1,2-3H resulted in the formation of a radioactive metabolite with a mobility slightly less than that of corticosterone in the chromatography system employed. Chemical identification of this metabolite was not attempted, although its mobility was similar to that of 18-hydroxy-deoxycorticosterone.

Radiochemical purity of the isolated corticosterone was estimated by formation of the 21-monoacetate. Chemical formation of the 21-monoacetate of corticosterone was achieved by treating the steroid with four drops of pyridine-acetic anhydride (1:1) for two hours at room temperature. The reaction mixture was then diluted with methanol and
evaporated to dryness under nitrogen. This step was repeated twice. The reaction product, after drying in vacuo, was chromatographed in the solvent system toluene-propylene glycol. The 21-monoacetate zone was excised, eluted with methanol, and assayed for radioactivity. Evidence for radiochemical homogeneity was provided by unaltered specific activity (cpm/µmole) throughout this purification procedure.

(iii) Radioassay method:

$^3$H activity or $^{14}$C activity was measured with a Nuclear-Chicago model 725 liquid scintillation counter. Samples of the radioactive steroid in methanol solution were pipetted into liquid scintillation vials and the methanol was removed in vacuo. The steroid was then dissolved in 5 ml of toluene containing PPO (0.4%), POPOP (0.005%), and absolute ethanol (1%). All radioactivity assays were performed in duplicate. The efficiency of the counting method for $^{14}$C was 86% and for $^3$H was 36-40%.

(j) Spectrophotometric Analysis of the Enzyme Preparation

The technique of difference spectoscopy was employed to assay the acetone powder enzyme preparation for cytochrome and flavin components. Difference spectra were measured in a Unicam SP 800 spectrophotometer with quartz cuvettes of 1 cm optical path. Unless otherwise stated, difference spectra were recorded at room temperature. The sample to be assayed was divided equally between two cuvettes and a baseline was recorded. After addition of appropriate agents to
the sample cuvette, the spectrum was then recorded. The baseline was subtracted from the change in light absorbance produced on addition of the agents to the sample cuvette and the resultant difference spectrum was plotted.

In a typical spectrophotometric assay of the enzyme preparation, 0.1 ml of propylene glycol, 2.0 ml of Tris-MgCl₂ buffer medium, and 3.9 ml of the enzyme preparation were combined and the mixture was divided equally between two cuvettes. Two types of difference spectra were then measured.

1. Reduced-oxidized difference spectra. After establishing a baseline from 600 to 400 nm, 1.0 μmole of NADPH in 0.05 ml of Tris-MgCl₂ buffer medium was added to the sample cuvette, and an equivalent amount of Tris-MgCl₂ buffer medium was added to the reference cuvette. The difference spectrum was then recorded. In the chemically produced difference spectra, a few crystals of sodium dithionite were added instead of NADPH to the sample cuvette.

2. Carbon Monoxide difference spectra. Prior to dividing the reaction mixture between two cuvettes, NADPH or dithionite was added to the reaction mixture to reduce the hemoproteins. The mixture was then divided equally between two cuvettes and a baseline recorded. Carbon monoxide was then gently bubbled through the sample cuvette solution for one minute and the difference spectrum was then recorded.

(k) Ammonium Sulfate Fractionation of the Enzyme Preparation

One gram of adrenal mitochondrial acetone powder was homogenized with 25 ml of a 0.154 M KCl solution and the enzyme
extract was obtained as previously described. This enzyme preparation was then fractionated with ammonium sulfate in a manner similar to that described by Kimura and Suzuki (59). The preparation was maintained at 4° C throughout the fractionation procedure.

The enzyme preparation was brought to 20% saturation by the addition of solid ammonium sulfate. The mixture was allowed to stand for ten minutes and then was centrifuged at 20,000 x g for thirty minutes. The supernatant was decanted, brought to 40% saturation with solid ammonium sulfate, allowed to stand for ten minutes, and then centrifuged at 20,000 x g for 30 minutes. The supernatant was again decanted and brought to 80% saturation with solid ammonium sulfate. After standing 10 minutes the mixture was centrifuged at 20,000 x g for 30 minutes and the supernatant was decanted.

The precipitates obtained between 0% and 20%, 20% and 40%, and 40% and 80% saturation with ammonium sulfate were redissolved in 1.8, 3.0, and 6.0 ml respectively of a 0.033 M Tris buffer solution containing 8.3 x 10^-4 M MgCl₂ and 0.1 M KCl. The dithionite reduced minus oxidized spectrum and the dithionite reduced carbon monoxide difference spectrum of each fraction were then determined.

(1) Studies on Cytochrome P-450 and P-420

(i) Effects of 11β-hydroxylase substrates, inhibitors (Dicumarol or Metopirone), and incubation conditions: Cytochrome P-450 and P-420 levels were measured under
experimental conditions and in the presence of agents similar to those employed to measure 11β-hydroxylation. It was found that the cytochrome P-450 content varied considerably between different enzyme preparations. For this reason control incubations were carried out simultaneously with incubations in the presence of steroid and/or of the inhibitors dicumarol and Metopirone.

Because the quantity of cytochrome P-450 in the enzyme preparation was relatively low, the total volume of reaction mixtures employed in these studies was three times greater than that employed in the kinetic studies. However the final concentration of each reaction component was the same. A typical reaction mixture for these studies contained: propylene glycol (with or without deoxycorticosterone) 0.15 ml; Tris-MgCl₂ buffer mixture, 2.1 ml; enzyme preparation, 3.9 ml; inhibitor (dicumarol or Metopirone) in 0.15 ml of the appropriate solvent. The contents of reaction flask were then pre-incubated at 37° C in a manner analogous to the 11β-hydroxylation studies. In some instances, NADPH in 0.15 ml of Tris-MgCl₂ buffer medium was added and the incubation continued. After the appropriate incubation period solid ammonium sulfate was added to give 20% saturation and the mixture was cooled to 4° C. The fraction precipitating between 20% and 40% ammonium sulfate saturation was then obtained as previously described. The precipitate was redissolved in 1.5 ml of a 0.033 M solution of Tris buffer medium containing $8.3 \times 10^{-4}$ M MgCl₂ and 0.1 M KCl. The
cytochrome P-450 and P-420 content of this mixture was then
determined in the following manner. The hemoproteins of each
mixture were reduced by addition of a few crystals of sodium
dithionite and the carbon monoxide difference spectrum was
determined as previously described. Cytochrome P-450 was
measured by the change in absorbance between 450 m\(\mu\) and 500 m\(\mu\). 
Cytochrome P-420 was measured by the change in absorbance be­
tween 420 m\(\mu\) and 500 m\(\mu\).

(ii) Substrate and inhibitor-induced spectral changes
in cytochrome P-450:

The enzyme preparation fraction precipitating between
20\% and 40\% saturation with ammonium sulfate was employed
for the studies on spectral changes induced in cytochrome
P-450 by deoxycorticosterone, dicumarol and Metopirone.
Because these spectral changes were of low intensity a more
concentrated solution of the 20\% to 40\% ammonium sulfate
precipitate was employed. Reaction mixtures for these
studies were set up in the following manner. The 20\% to
40\% ammonium sulfate precipitate obtained from 8 ml of the
enzyme preparation was dissolved in 1.6 ml of a 0.0375 M
Tris buffer solution containing 9.4 x 10^{-4} M MgCl\(_2\) and
0.096 M KCl. The mixture was divided equally between two
cuvettes and a baseline from 500 m\(\mu\) to 375 m\(\mu\) was recorded.
Deoxycorticosterone dissolved in 0.02 ml of propylene glycol
was added to the sample cuvette; the reference cuvette re­
ceived the same quantity of propylene glycol. The difference
spectrum was then recorded. The difference spectrum induced by the addition of deoxycorticosterone was found to be stable throughout the period of time during which these experiments were conducted.

The ability of the 11β-hydroxylase inhibitors dicumarol and Metopirone to induce spectral changes in cytochrome P-450 was measured in the same manner as that employed for deoxycorticosterone. The effects of dicumarol and Metopirone on the deoxycorticosterone-induced spectral changes were measured by two procedures:

1. After recording the deoxycorticosterone-induced difference spectrum, dicumarol or Metopirone in 0.01 ml of the appropriate solvent was added to both the sample and reference cuvettes. The difference spectrum was then again recorded. A control experiment was carried out in which 0.01 ml of solvent alone was added to each cuvette. This addition was found to have no effect on the magnitude of the deoxycorticosterone-induced difference spectrum.

2. Prior to the addition of deoxycorticosterone, dicumarol or Metopirone dissolved in 0.01 ml of the appropriate solvent was added to both the sample and reference cuvettes. A baseline from 500 mμ to 375 mμ was then recorded. Deoxycorticosterone dissolved in 0.02 ml of propylene glycol was added to the sample cuvette and 0.02 ml of propylene glycol was added to the reference cuvette. The difference spectrum was then recorded.
(m) Studies on Bovine Adrenal Mitochondrial Lipids

(i) Acetone-extracted lipid fraction:

The acetone-extracted lipid fraction refers to the material that is extracted into acetone during the preparation of the adrenal mitochondrial acetone powder. The acetone was removed from the fraction under reduced pressure in a warm water bath. All water was removed from the lipid fraction by azeotropic distillation with absolute ethanol. The dry material was then ground into a fine powder with a mortar and pestle.

For studies on the effect of this lipid fraction on NADPH oxidation, 11β-hydroxylation, or on cytochrome P-450, the dried powder was suspended in a 0.1 M Tris buffer solution containing $2.5 \times 10^{-3}$ M $\text{MgCl}_2$, pH 7.4. The lipid concentration was usually 10 mg/ml. The suspension was then sonicated in a Bronwill 20-kc sonic oscillator for 20 minutes at 4° C to disperse the lipid material. Such treatment orients the phospholipids to form micelles (109,110). In comparative studies with the commercial phospholipid preparation asolectin, a dispersed suspension of this lipid was prepared in a similar manner. The lipid suspensions were prepared immediately prior to use in incubation mixtures.

(ii) Preliminary fractionation of the acetone-extracted lipid material:

A preliminary fractionation of the acetone-extracted lipid material was carried out as follows. Five g of the
dried lipid were extracted with 50 ml of petroleum ether (b.p. 40° - 60° C) by continuous shaking of the lipid-solvent mixture for two hours with a Burell wrist agitator. The suspension was then centrifuged, the supernatant decanted, and the precipitate was re-extracted with an additional 50 ml of petroleum ether. The combined petroleum ether extract was concentrated to a volume of 20 ml under reduced pressure and the concentrate was poured, with stirring, into 200 ml of acetone previously chilled to -20° C. The mixture was allowed to stand overnight at -20° C. The precipitate that formed was removed by centrifugation and the acetone was evaporated from the supernatant under reduced pressure. A portion of each fraction obtained in this separation procedure was examined for its effect on NADPH oxidation and 11β-hydroxylation. Each sample was dried in vacuo, and an equivalent amount of the dried material from each fraction was dispersed in Tris-MgCl₂ buffer medium by ultrasonic treatment.

(iii) Extraction of the total lipids of adrenal mitochondria:

The procedure employed for the extraction and isolation of the adrenal mitochondrial lipids was similar to the method described by Crane et al. (111) for the isolation of coenzyme Q from beef heart mitochondria.

Seventy g wet weight of mitochondria, obtained from 1000 g of whole beef adrenals, were suspended in a 0.25 M solution of sucrose to give a total volume of 150 ml. The subsequent
extraction and fractionation of lipids were carried out at room temperature. The mitochondrial suspension was poured into 1,500 ml of an ethanol-ether mixture (3:1 v/v) and the mixture was stirred continuously for one hour. Solids were filtered off on a Buchner funnel and twice re-extracted with ethanol-ether by the same procedure. The combined ethanol-ether extract was evaporated under reduced pressure in a warm water bath until the total volume was 250 ml. This aqueous ethanol suspension (250 ml) was then extracted with petroleum ether, b.p. 40° - 60° C, (3 x 150 ml) and the combined petroleum ether extract was reduced to a volume of 15 ml under reduced pressure. This extract was then poured, with stirring, into 300 ml of acetone and the mixture was stored at -20° C overnight. The acetone suspension was then filtered in the cold to remove precipitated phospholipid. The filtrate was evaporated under reduced pressure and the residue was redissolved in 20 ml of absolute ethanol with warming. The ethanol solution was stored overnight at -20° C and the white crystalline material obtained (cholesterol) was then removed by centrifugation.

Each fraction obtained by this procedure was examined for its effect on NADPH oxidation by the enzyme preparation. An equivalent percentage of each fraction was removed, evaporated to dryness and the material redissolved in ethanol. A portion of each ethanol fraction was then added to reaction mixtures employed in the measurement of NADPH oxidation.
(iv) Silica gel column chromatography of the lipid fraction soluble in ethanol at -20°C:

A silica gel column was prepared by mixing approximately 13 g of silica gel (Davison grade 923, mesh size 100-200) with hexane followed by pouring the slurry into a glass column half-filled with hexane. The silica gel was allowed to settle and the excess hexane was drained off. The final dimensions of the silica gel column were 1 cm x 19 cm. The -20°C ethanol-soluble fraction obtained by fractionation of the total mitochondrial lipids from 70 g wet weight of adrenal mitochondria was evaporated to dryness under reduced pressure, redissolved in 15 ml of hexane, and added to the column. The column was then developed with the following solvents or solvent combinations: 250 ml of hexane, 50 ml of hexane-chloroform (19:1), 50 ml of hexane-chloroform (9:1), 50 ml of hexane-chloroform (4:1), 50 ml of hexane-chloroform (1.5:1), 150 ml of chloroform, and 100 ml of methanol. Fifty ml eluate fractions were collected. The solvent was removed from each fraction under reduced pressure and each residue was redissolved in 5.0 ml of 95% ethanol. Equivalent portions of each fraction were then assayed for their effect on NADPH oxidation.

A portion of each column eluate was applied to a thin-layer chromatography plate of silica gel G. The chromatography plate was developed with benzene. A sample of commercial coenzyme Q₁₀ (Mann Research Laboratories) was run as a standard. The developed chromatogram was examined for
ultra-violet absorbing zones and was then sprayed with a solution of leucomethylene blue. Zones capable of undergoing reduction appeared as blue spots. Reduced (leuco) methylene blue was prepared by the method of Crane and Dilley (112). 50 mg of methylene blue were dissolved in 50 ml of 95% ethanol. 0.5 g of zinc dust and 0.5 ml of glacial acetic acid were added, the mixture swirled until colourless and then filtered through glass wool. This reagent was then sprayed on the thin-layer chromatography plate.

(v) Saponification of the adrenal mitochondrial acetone powder:

The possible presence of bound lipoquinones in the mitochondrial acetone powder was examined by saponification of the preparation according to the method of Gale et al. (113). Four g of adrenal mitochondrial acetone powder, 2.0 g of pyrogallol, 8 g of sodium hydroxide, 40 ml of methanol and 30 ml of water were added to a 250 ml round bottom flask and the mixture was refluxed for one hour. After cooling slightly, the still warm reflux mixture was extracted with hexane (3 x 150 ml). The combined hexane extract was concentrated to 50 ml under reduced pressure. The concentrate was then washed with water (3 x 50 ml), dried over anhydrous sodium sulfate, filtered, and evaporated to dryness under reduced pressure. This material was then assayed for lipoquinones according to the procedure outlined by Bragg and Polglase (114). The hexane-extracted material was dissolved in 5 ml of iso-octane and applied to a Magnesol-Celite (5:1) column, the dimensions of which were 1 cm x 3 cm. The column was
eluted with the following solvents or solvent combinations:
20 ml of iso-octane, 20 ml of ether-iso-octane (1:99), 2 x
20 ml of ethanol-iso-octane (1:99), and 20 ml of acetone.
Each eluate was evaporated to dryness, the material redis­solved in 2 ml of iso-octane and then assayed spectrophoto­metrically from 340 to 230 μm for the presence of lipoquinones.
PART I  GENERAL PROPERTIES OF THE 11β-HYDROXYLASE EXTRACTED FROM A BOVINE ADRENAL MITOCHONDRIAL ACETONE POWDER

The enzyme system which catalyzes the introduction of a hydroxyl group into the 11β position of the steroid nucleus is located in the mitochondrial fraction of adrenocortical tissue. The 11β-hydroxylase has been isolated by a variety of methods (31-33) from adrenal tissue. The enzyme employed in the current studies was extracted from a bovine adrenal mitochondrial acetone powder with a 0.154 M solution of KCl. The general properties of the system isolated in this manner were studied.

RESULTS

(a) NADPH Requirement of the 11β-Hydroxylase

The steroid 11β-hydroxylase is a member of the general group of enzymes termed mixed function oxidases that catalyze the introduction of an atom of molecular oxygen into the substrate molecule concomitant with the oxidation of NADPH. The dependence of 11β-hydroxylation on NADPH in the enzyme preparation employed in our studies is shown in Table I. No conversion of deoxycorticosterone to corticosterone occurs in the absence of added NADPH. Indeed the data indicate that extensive conversion takes place only at high molar ratios of NADPH to deoxycorticosterone. For example, 10 μmoles of NADPH are required for the formation of 0.37 μmole of corticosterone. That no corticosterone can be
TABLE I

Effect of increasing quantities of NADPH on the 11β-hydroxylation of deoxycorticosterone

<table>
<thead>
<tr>
<th>NADPH added (µmoles)</th>
<th>Deoxycorticosterone recovered (µmole)</th>
<th>Corticosterone synthesized (µmole)</th>
<th>Percent conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.430</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.214</td>
<td>0.213</td>
<td>44</td>
</tr>
<tr>
<td>7.5</td>
<td>0.078</td>
<td>0.325</td>
<td>67</td>
</tr>
<tr>
<td>10</td>
<td>0.014</td>
<td>0.365</td>
<td>75</td>
</tr>
</tbody>
</table>

Each reaction flask contained: 0.484 µmole of deoxycorticosterone dissolved in 0.10 ml of propylene glycol; 1.50 ml of Tris-MgCl₂ buffer; 3 ml of enzyme preparation (1 g of mitochondrial acetone powder extracted with 10 ml of a 0.154 M KCl solution); NADPH dissolved in 0.5 ml of Tris-MgCl₂ buffer. The total volume was 5.1 ml. After 90 minutes of incubation, reactions were terminated by the addition of ethyl acetate (15 ml) to each flask.
detected in the absence of added NADPH excludes the presence of endogenous corticosterone in the 11β-hydroxylase preparation.

Péron and associates (115,116) found in studies with intact mitochondria that NADPH supported only a relatively low rate of 11β-hydroxylation when compared with that by Krebs cycle intermediates. Similarly Harding et al. (117) found that succinate and malate supported a much higher rate of 11β-hydroxylation than did NADPH. Both groups of workers suggested that the function of the tricarboxylic acid cycle intermediates was to generate intramitochondrial NADPH required for 11β-hydroxylation. They attributed the inability of exogenous NADPH to support 11β-hydroxylation to the impermeability of mitochondria to reduced pyridine nucleotides (115-119). Indeed Péron et al. (116,120) showed that NADPH-supported 11β-hydroxylation could be stimulated by the addition of calcium and concluded that mitochondrial swelling resulting from the addition of calcium allowed exogenous NADPH to enter the mitochondria and thus support 11β-hydroxylation. The enzyme preparation used in our studies was an extract of a mitochondrial acetone powder. Therefore the impermeability of NADPH would not be a limiting factor and could not account for the high concentrations of NADPH required by this preparation for 11β-hydroxylation.

The requirement of the enzyme preparation for high concentrations of NADPH suggested that the cofactor was being utilized in reactions other than 11β-hydroxylation. Oxidation of NADPH by the enzyme preparation in both the absence and
presence of steroid substrate was therefore examined. The results are shown in Fig. 4. It is apparent that the enzyme preparation catalyzes the rapid disappearance of the reduced coenzyme in the absence of steroid substrate. Addition of deoxycorticosterone to the reaction mixture results in an increased rate of NADPH oxidation. The "background" oxidation of NADPH in the absence of steroid is responsible for 83% of the total NADPH oxidized in the presence of steroid during a 90 minute incubation period and likely accounts for the high molar ratios of NADPH to steroid that are required for significant hydroxylation.

11β-hydroxylation of deoxycorticosterone was measured throughout the period of NADPH oxidation shown in Figure 4. The percent conversion of deoxycorticosterone to corticosterone as a function of time is shown in Table II. Hydroxylation proceeds most rapidly during the first 45 minutes of incubation. A maximum of 70% conversion of deoxycorticosterone to corticosterone is achieved after 60 minutes of incubation and no significant increase is observed after this time even though NADPH oxidation is still proceeding (Fig. 4). The decreased rate of corticosterone formation indicates that possibly some other factor involved in 11β-hydroxylation is becoming rate limiting, or that an enzyme in the 11β-hydroxylase system is unstable to prolonged incubation. The decreased rate of 11β-hydroxylation may also be the result of inhibition of the reaction by the product corticosterone.

Comparison of the NADPH oxidation directly attributable
Fig. 4. Oxidation of NADPH in the presence and absence of deoxycorticosterone.

Incubations were carried out as described in Table II. Prior to the addition of ethyl acetate, 0.2 ml of each reaction mixture was removed, diluted to 4 ml with water, and the absorption at 340 m\(\mu\) was measured. NADPH oxidation in the absence of deoxycorticosterone was measured after 0, 15, 30, 45, 60, and 90 minutes of incubation by removing 0.2 ml portions of a reaction mixture containing 0.1 ml propylene glycol instead of deoxycorticosterone.

Control (○); plus deoxycorticosterone (○).
### TABLE II

11β-hydroxylation of deoxycorticosterone as a function of time

<table>
<thead>
<tr>
<th>Incubation time (minutes)</th>
<th>Deoxycorticosterone recovered (µmole)</th>
<th>Corticosterone synthesized (µmole)</th>
<th>Percent conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.372</td>
<td>0.246</td>
<td>30.8</td>
</tr>
<tr>
<td>30</td>
<td>0.251</td>
<td>0.375</td>
<td>47.1</td>
</tr>
<tr>
<td>45</td>
<td>0.106</td>
<td>0.517</td>
<td>64.8</td>
</tr>
<tr>
<td>60</td>
<td>0.039</td>
<td>0.563</td>
<td>70.5</td>
</tr>
<tr>
<td>75</td>
<td>0.030</td>
<td>0.568</td>
<td>71.3</td>
</tr>
<tr>
<td>90</td>
<td>0.036</td>
<td>0.573</td>
<td>71.8</td>
</tr>
</tbody>
</table>

Each reaction flask contained: 0.800 µmole of deoxycorticosterone dissolved in 0.1 ml of propylene glycol; 1.50 ml of Tris-MgCl₂ buffer; 3 ml of enzyme preparation (1 g of mitochondrial acetone powder extracted with 10 ml of a 0.154 M KCl solution); 5.5 µmoles of NADPH dissolved in 0.5 ml of Tris-MgCl₂ buffer. The total volume was 5.1 ml. After 15, 30, 45, 60 or 90 minutes of incubation, reactions were terminated by the addition of ethyl acetate (15 ml) to each flask.
to 11β-hydroxylation, i.e., the increase in NADPH oxidation produced by the addition of deoxycorticosterone (Fig. 4), and actual production of corticosterone (Table II) was made. After 15, 30, and 45 minutes of incubation, the amount of corticosterone produced was 0.25, 0.38, and 0.52 µmole respectively. The increased amount of NADPH oxidized upon addition of deoxycorticosterone was 0.63, 0.90, and 1.3 µmoles after these same incubation periods. Thus the ratio of NADPH oxidized to corticosterone produced was 2.5, 2.4, and 2.9 after 15, 30, and 45 minutes of incubation. The stoichiometry of this 11β-hydroxylation reaction differs from that of the microsomal steroid 21-hydroxylase. Cooper et al. (40) showed that in the hydroxylation of steroids at C-21, one mole of NADPH was oxidized for each mole of C-21 hydroxylated steroid formed.

In order to ascertain the relationship between NADPH oxidation and 11β-hydroxylation, the effects of some known electron transport inhibitors on these processes were examined. The data in Table III illustrate the effects of these inhibitors on NADPH oxidation in the absence of steroid substrate. Potassium cyanide has no effect on the oxidation of NADPH. This compound is a well-established inhibitor of cytochrome oxidase, the terminal oxidase of the electron transport chain. Lack of inhibition of NADPH oxidation by potassium cyanide suggests that oxidation of NADPH is not proceeding via the classical electron transport chain. Moreover, cytochrome oxidase is readily destroyed by acetone
### TABLE III

**Effect of inhibitors on NADPH oxidation**

<table>
<thead>
<tr>
<th>Incubation time (minutes)</th>
<th>Percent of control activity</th>
<th>Inhibitor added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KCN</td>
</tr>
<tr>
<td>5</td>
<td>104</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>89</td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>89</td>
</tr>
</tbody>
</table>

Appropriate reaction flasks contained: 1.0 ml of Tris-MgCl₂ buffer; 2.0 ml of enzyme preparation (1 g of mitochondrial acetone powder extracted with 15 ml of a 0.154 M KCl solution); 0.1 ml of a 3.0 x 10⁻² M solution of potassium cyanide, final concentration, 9.5 x 10⁻⁴ moles/l; 0.04 ml of a 1.8 x 10⁻³ M solution of antimycin A, final concentration, 2.3 x 10⁻⁵ moles/l; 0.1 ml of a 2.0 x 10⁻² M solution of amytal, final concentration, 6.4 x 10⁻⁴ moles/l; or 0.05 ml of a 1.0 x 10⁻² M solution of dicumarol, final concentration, 1.6 x 10⁻⁴ moles/l. The volume of each reaction mixture was adjusted to 3.15 ml with water and 1.0 μmole of NADPH dissolved in 0.05 ml of Tris-MgCl₂ buffer was added at zero time.
treatment (31,121) and is unlikely to be present in an acetone powder of beef adrenal mitochondria. Two other inhibitors tested, amytal and antimycin A have no effect on NADPH oxidation, confirming that the electron transport chain is not functioning in this system. Dicumarol, an uncoupler of oxidative phosphorylation (122-124), produces a slight increase in the rate of NADPH oxidation. Increased respiration by mitochondria in the presence of dicumarol, as well as other uncoupling agents has been reported (39,125). Whether the increase in NADPH oxidation demonstrated in Table III is related to the stimulation of respiration reported in studies with intact mitochondria is uncertain. Alternatively, dicumarol may be functioning as an electron acceptor in this system. Examination of the molecular structure of dicumarol (Fig. 3) indicates positions that could possibly undergo reduction or hydroxylation.

The effects of these inhibitors on 11β-hydroxylation are shown in Table IV. Neither amytal nor antimycin A produces any inhibition of 11β-hydroxylation. Potassium cyanide produces a slight inhibition. Studies by several groups (70,115,117,126) have shown that NADPH-supported 11β-hydroxylation is generally insensitive to cyanide, although Fonzo et al. (126) did find slight inhibition produced at higher concentrations of cyanide (2-4 mM) when the concentration of NADPH became rate-limiting. Schenkman et al. (97) have observed that potassium cyanide could bind to the microsomal hydroxylase of rat liver. The concentration at which potassium cyanide interacted with the microsomal hydroxylase was considerably higher than the substrate concentration


**TABLE IV**

<table>
<thead>
<tr>
<th>Inhibitor added</th>
<th>Final concentration (moles/1)</th>
<th>Percent of control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCN</td>
<td>$1.0 \times 10^{-3}$</td>
<td>77</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>$2.5 \times 10^{-5}$</td>
<td>98</td>
</tr>
<tr>
<td>Amytal</td>
<td>$6.6 \times 10^{-4}$</td>
<td>100</td>
</tr>
<tr>
<td>Dicumarol</td>
<td>$3.2 \times 10^{-4}$</td>
<td>0</td>
</tr>
<tr>
<td>Dicumarol</td>
<td>$1.9 \times 10^{-4}$</td>
<td>11</td>
</tr>
<tr>
<td>Dicumarol</td>
<td>$9.5 \times 10^{-5}$</td>
<td>43</td>
</tr>
</tbody>
</table>

Appropriate reaction flasks contained: 0.76 μmole of deoxycorticosterone dissolved in 0.05 ml of propylene glycol; 1.50 ml of Tris-MgCl₂ buffer; 3 ml of enzyme solution (1 g of mitochondrial acetone powder extracted with 15 ml of a 0.154 M KCl solution); inhibitor solutions as described in Table III to give the final concentration indicated in the table. The volume of each reaction mixture was adjusted to 5.0 ml with water and 4 μmoles of NADPH dissolved in 0.2 ml of Tris-MgCl₂ buffer was added at zero time. After 60 minutes of incubation, reactions were terminated by the addition of ethyl acetate (15 ml) to each flask.
required for a similar interaction with this system. The slight inhibition of 11β-hydroxylation produced by cyanide under the present experimental conditions (Table IV) suggests that potassium cyanide can interact to a limited extent with the 11β-hydroxylase.

The data in Table IV show that dicumarol inhibits the 11β-hydroxylation of deoxycorticosterone. One hundred percent inhibition of corticosterone formation is achieved at a dicumarol concentration of $3.2 \times 10^{-4}$ moles/l. This inhibition is obtained in spite of the fact that neither the electron transport chain nor high energy compounds in the form of ATP are required for 11β-hydroxylation when NADPH is employed as the cofactor. It is apparent that the inhibition of 11β-hydroxylation by dicumarol under these conditions is not a function of its ability to uncouple oxidative phosphorylation, and that an alternate mechanism is involved.

(b) Kinetic Study of 11β-Hydroxylation

Experiments to establish the kinetics of 11β-hydroxylation by the enzyme system extracted from the acetone powder of beef adrenal mitochondria were carried out. The formation of corticosterone as a function of incubation time at two substrate concentrations is shown in Figure 5. At the higher substrate concentration (22 µmoles/l) the rate of 11β-hydroxylation is constant during the first six minutes of incubation. At this point 17% of the substrate deoxycortico-
Fig. 5. The rate of 11β-hydroxylation as a function of deoxycorticosterone concentration.

Each reaction flask contained: deoxycorticosterone-1, 2-\(^{3}\)H (specific activity 2.15 x 10\(^{7}\) cpm/umole) dissolved in 0.05 ml of propylene glycol; 1.35 ml of Tris-MgCl\(_2\) buffer; 2.6 ml of enzyme preparation; and 0.1 ml of a dilute alcoholic KOH solution. Reaction mixtures were pre-incubated for 5 minutes. NADPH (3.3 μmoles) dissolved in 0.2 ml of Tris-MgCl\(_2\) buffer was added to each reaction flask at zero time. 1.0 ml samples were removed from each reaction flask after 3, 6, 9, and 12 minutes of incubation and pipetted into ethyl acetate (2 ml) to terminate the reactions.

Deoxycorticosterone final concentration, μmoles/l: 4.4 (♀); 22.2 (♂).
sterone has been converted to corticosterone. At the lower substrate concentration (4.4 μmoles/l) the rate of 11β-hydroxylation rapidly becomes nonlinear, due to utilization of steroid substrate. At this concentration, steroid substrate very quickly becomes the rate-limiting factor in 11β-hydroxylation. A plot of the initial velocity of 11β-hydroxylation against substrate concentration (Fig. 6) produces a typical hyperbolic saturation curve. Kinetic presentation of this data (Fig. 6) indicates that the $K_m$ for 11β-hydroxylation of deoxy­corticosterone is 5.5 μmoles/l. This value is in close agreement with that determined by Sharma et al. (33). They obtained a $K_m$ for deoxycorticosterone of 8.5 μmoles/l employing a lyophilized enzyme preparation obtained from a 105,000 x g supernatant of sonicated beef adrenal mitochondria. However, it is lower than the $K_m$ of 20 μmoles/l obtained by Dominguez and Samuels (45) employing a whole homogenate of rat adrenal glands. This discrepancy may be due to the different enzyme preparations employed; that is a mitochondrial extract vs. an adrenal homogenate.

(c) **Spectrophotometric Studies on the Enzyme Preparation**

Spectrophotometric examination of adrenal mitochondria has shown that the mitochondria of this tissue possess the components of the classical respiratory chain (39,117,127). In addition, there is present in these mitochondria a second type of respiratory chain that transfers electrons from NADPH to cytochrome P-450 for the 11β-hydroxylation of
Fig. 6. [S]/v versus [S] plot for the 11β-hydroxylation of deoxycorticosterone.

Each reaction flask contained: deoxycorticosterone-1, 2\(^{-3}\)H (specific activity 6.24 \times 10^7 cpm/μmole) dissolved in 0.05 ml of propylene glycol; 0.70 ml of Tris-MgCl\(_2\) buffer; 1.30 ml of enzyme preparation; and 0.05 ml of a dilute alcoholic KOH solution. Reaction mixtures were pre-incubated for 8 minutes. NADPH (1.6 μmoles) dissolved in 0.05 ml of Tris-MgCl\(_2\) buffer was added to each flask at zero time. After 90 seconds of incubation a sample (1.0 ml) was removed from each flask and pipetted into ethyl acetate (2 ml) to terminate the reaction.
deoxycorticosterone (39,56,127). Spectrophotometric examina-
tion of the enzyme preparation (extracted from an acetone
powder of bovine adrenal mitochondria) employed in our studies
was therefore undertaken.

(i) Reduced - oxidized and carbon monoxide difference
spectra of the enzyme preparation.

The reduced minus oxidized difference spectrum of the
enzyme preparation is shown in Figure 7. These spectra are
produced both by enzymatic reduction with NADPH and by
chemical reduction with sodium dithionite (Na₂S₂O₄). The
difference spectrum produced by reduction with NADPH ex-
hibits absorption maxima at 570, 545, and 419 μm and a
trough at 460 - 470 μm. The absorption maximum at 419 μm
is similar to that of cytochrome c (85), but the maxima at
570 and 545 μm do not correspond to the absorption maxima
exhibited by any of the normal respiratory chain cytochromes
in this region of the spectrum (85,127). The formation of
the absorption maximum at 419 μm upon addition of NADPH may
indicate the presence of a NADPH-cytochrome c reductase in
the enzyme preparation. No absorption maxima corresponding
corresponding to cytochromes a or a₃, cytochrome b, or
cytochrome P-420 are observed. As mentioned previously, it
is quite likely that cytochromes a and a₃ are destroyed
during preparation of the acetone powder. The decrease in
absorbance in the region 460 - 470 μm suggests the reduction
of a flavoprotein present in the enzyme preparation (85).
Fig. 7. Reduced minus oxidized difference spectrum of the mitochondrial acetone powder enzyme preparation reduced with NADPH or sodium dithionite.

Each cuvette contained: 0.05 ml of propylene glycol; 1.0 ml of Tris-MgCl₂ buffer; and 1.95 ml of enzyme preparation. NADPH (1.1 µmoles), dissolved in 0.05 ml of Tris-MgCl₂, or sodium dithionite was added to the sample cuvette and the difference spectrum was recorded.

NADPH reduced difference spectrum (-- -- --); dithionite reduced difference spectrum (-----).
Chemical reduction of the enzyme preparation with sodium dithionite produces a difference spectrum with absorption maxima at 556, 518, and 430 m\(\mu\) and with a trough at 460 m\(\mu\). The maxima present in the NADPH-reduced difference spectrum are no longer evident, being obscured by the more prominent chemically produced maxima. The dithionite-produced absorption maximum at 430 m\(\mu\) has twice the intensity of the NADPH-produced absorption maximum at 419 m\(\mu\). The dithionite-produced difference spectrum is identical to the difference spectrum of hemoglobin produced by dithionite and most probably represents hemoglobin contamination of the enzyme preparation (84,117,127). Cytochrome \(b\) may also be contributing to this dithionite-produced difference spectrum (85,127).

The carbon monoxide difference spectra of the enzyme preparation reduced with both NADPH and dithionite are shown in Figure 8. The two spectra are identical with absorption maxima at 470, 534, 457 - 460 and 418 m\(\mu\), and correspond to the carbon monoxide difference spectrum of a hemoglobin solution treated in the same manner. There is no absorption maximum at 450 m\(\mu\) corresponding to cytochrome P-450. This absorption band has been shown to be a distinct feature of the carbon monoxide difference spectrum of adrenal mitochondria (56,70,85,86,127). In addition no absorption maxima corresponding to the carbon monoxide complex of cytochromes \(a\) and \(a_3\) are observed.
Fig. 8. Carbon monoxide difference spectrum of the mitochondrial acetone powder enzyme preparation reduced with NADPH or sodium dithionite.

Incubations were carried out as described in Fig. 7. NADPH (1.1 μmoles) dissolved in 0.05 ml Tris-MgCl₂, or sodium dithionite was added to both the sample and reference cuvettes. Carbon monoxide was bubbled through the sample cuvette solution for 60 seconds and the difference spectrum was recorded. NADPH reduced (———); sodium dithionite reduced (-----).
(ii) Ammonium sulfate fractionation of the enzyme preparation.

Because of the interference by hemoglobin in attempts to identify cytochrome P-450 as a component of the enzyme preparation employed in the present studies, the ammonium sulfate fractionation procedure of Kimura and Suzuki (59) was employed in order to separate any cytochrome P-450 that may be present in the enzyme preparation from hemoglobin. By this procedure cytochromes P-450 and P-420 can be obtained in a fraction precipitating between 20% and 40% saturation with ammonium sulfate. A similar procedure has been reported by Sweat and Young (128).

The reduced minus oxidized difference spectrum and the carbon monoxide difference spectrum of the fractions precipitating between 0% and 20%, 20% and 40%, and 40% and 80% saturation with ammonium sulfate are shown in Figures 9, 10, and 11 respectively. Both the reduced minus oxidized difference spectrum (Fig. 9a) and the carbon monoxide difference spectrum (Fig. 9b) of the fraction precipitating between 0% and 20% saturation with ammonium sulfate are identical to those obtained with hemoglobin.

The reduced minus oxidized difference spectrum of the fraction precipitating between 20% and 40% saturation with ammonium sulfate exhibits absorption maxima at 555 - 557, 532, and 427 μm (Fig. 10a). These absorption maxima correspond closely to those exhibited in the difference spectrum of cytochrome P-420 of rabbit liver microsomes reported by Omura
Fig. 9a. Dithionite reduced minus oxidized difference spectrum of the 0-20% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

The 0-20% ammonium sulfate fraction obtained from 25 ml of enzyme preparation (1 g mitochondrial acetone powder extracted with 25 ml of a 0.154 M KCl solution) was dissolved in 1.8 ml of a solution made up of Tris-MgCl$_2$ buffer (0.6 ml) and 0.154 M KCl (1.2 ml) and divided equally between two cuvettes. A few crystals of sodium dithionite were added to the sample cuvette and the difference spectrum was recorded.

Fig. 9b. Dithionite reduced carbon monoxide difference spectrum of the 0-20% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

Sodium dithionite also was added to the reference cuvette of the experiment described in Fig. 9a. Carbon monoxide was bubbled through the sample cuvette solution for 60 seconds and the difference spectrum was recorded.
Fig. 10a. Dithionite reduced minus oxidized difference spectrum of the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

The difference spectrum was determined as described in Fig. 9a. The precipitate obtained between 20% and 40% ammonium sulfate saturation was dissolved in 3.0 ml of the Tris-KCl buffer mixture.

Fig. 10b. Dithionite reduced carbon monoxide difference spectrum of the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

The difference spectrum was determined as described in Fig. 9b.
Fig. 11a. Dithionite reduced minus oxidized difference spectrum of the 40-80% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

The difference spectrum was determined as described in Fig. 9a. The precipitate obtained between 40% and 80% saturation with ammonium sulfate was dissolved in 6.0 ml of the Tris-KCl buffer mixture.

Fig. 11b. Dithionite reduced carbon monoxide difference spectrum of the 40-80% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

The difference spectrum was determined as described in Fig. 9b.
and Sato (88,89). It is also possible that hemoglobin is contributing to this spectrum. The absorption maxima exhibited by cytochrome P-420 and hemoglobin are quite similar although the soret band for cytochrome P-420 is at 427 μm while that for hemoglobin is at 430 μm. Also present in the reduced minus oxidized difference spectrum of the fraction precipitating between 20% and 40% saturation with ammonium sulfate is a trough with a maximum at 456 μm. This trough is produced by reduction of flavoprotein and may represent the NADPH reductase (adrenodoxin reductase) that is a component of the 11β-hydroxylase pathway (56,57). Kimura and Suzuki (59) have shown that this flavoprotein precipitates between 20% and 40% saturation with ammonium sulfate.

The carbon monoxide difference spectrum of the fraction precipitating between 20% and 40% saturation with ammonium sulfate shows an intense absorption band at 450 μm indicating the presence of cytochrome P-450 in this fraction (Fig. 10b). Also present are absorption maxima at 572, 536, and 418 μm, which may represent cytochrome P-420, although these maxima are at wavelengths slightly shorter than those reported by Omura and Sato (88,89) for cytochrome P-420 of rabbit liver microsomes. Again hemoglobin may be contributing to this absorption spectrum. Cytochrome P-420 is not a normal constituent of the adrenal mitochondria, but is produced by decomposition of cytochrome P-450 (88-93). Our experiments indicate that cytochrome P-420 is present in the acetone powder of adrenal mitochondria.
The reduced minus oxidized difference spectrum of the fraction precipitating between 40% and 80% saturation with ammonium sulfate exhibits absorption maxima at 592, 556, 520, and 434 μm (Fig. 11a). A trough at 460 μm is apparent, indicating the presence of a flavoprotein in this fraction. Sweat and Young (128) have obtained a purified flavoprotein from a fraction precipitated by 70% saturation with ammonium sulfate, but found that it did not enhance 11β-hydroxylation. Cammer and Estabrook (85) have suggested that the decrease in absorbance at about 460 μm is possibly due to reduction of nonheme iron protein of the 11β-hydroxylase pathway as well as flavoprotein. Kimura and Suzuki (59) have demonstrated that the nonheme iron component is present in the fraction precipitating between 60% and 80% saturation with ammonium sulfate. Therefore both flavoprotein and nonheme iron may be contributing to the trough at 460 μm (Fig. 11a).

The carbon monoxide difference spectrum of the fraction precipitating between 40% and 80% saturation with ammonium sulfate is complex, having absorption maxima at 608, 570, 533, 422, and 412 μm and a pronounced minimum at 436 μm (Fig. 11b). These absorption maxima do not correspond to those of the carbon monoxide complex of any previously described cytochrome and perhaps represent carbon monoxide complexes of cytochrome degradation products formed during preparation of the mitochondrial acetone powder or to denaturation of hemoproteins such as cytochrome b to a form capable of binding carbon monoxide (90). Hemoglobin is also likely to be present in this
ammonium sulfate fraction.

Omura et al. (57) have carried out an ammonium sulfate fractionation of an 11β-hydroxylase prepared from sonicated adrenal mitochondria. They found that the 60-80% ammonium sulfate fraction contained nonheme iron protein, flavoprotein, cytochrome c, and a large amount of hemoglobin. The complex spectra of the 40-80% ammonium sulfate fraction (Figs. 11a and 11b) obtained in the present studies also indicates the presence of several components in this fraction.

(d) 11β-Hydroxylase from Sonicated Adrenal Mitochondria

Enzyme systems capable of introducing a hydroxyl function into the 11β position of the steroid nucleus have been prepared by various methods (31-33). Sharma et al. (33) have reported that the 11β-hydroxylase extracted from an acetone powder of beef adrenal mitochondria has a lower activity than the enzyme system solubilized by ultrasonic treatment of adrenal mitochondria. Therefore studies with an 11β-hydroxylase prepared by the method of Sharma et al. were undertaken in order to compare the properties of this system with the acetone powder preparation.

(i) NADPH oxidation and 11β-hydroxylation by the sonicated adrenal mitochondria preparation.

NADPH is oxidized by the enzyme prepared from sonicated mitochondria in the absence of added steroid substrate (Fig. 12), the rate of oxidation being proportional to the amount of enzyme present. Upon addition of excess quantities of
Fig. 12. NADPH oxidation by an enzyme preparation obtained by ultracentrifugation of sonicated adrenal mitochondria.

Each reaction flask contained: 0.05 ml of propylene glycol; 1.0 ml of Tris MgCl₂ buffer; 2.0 ml of a 0.154 M solution of KCl; and 15, 20, or 30 mg of the lyophilized enzyme preparation. 0.9 µmole of NADPH dissolved in 0.2 ml of Tris-MgCl₂ buffer was added at zero time.

Amount of enzyme preparation added (mg): 15 (○); 20 (▲); 30 (○); 20, plus 0.6 µmole of deoxycorticosterone (▲).
deoxycorticosterone (200 µg), no significant increase in the rate of oxidation of NADPH is observed (Fig. 12). Thus this system differs in this respect to the acetone powder preparation where addition of steroid substrate produces a substantial increase in the rate of NADPH oxidation (Fig. 4). The sonicated adrenal mitochondrial enzyme preparation does catalyze the 11β-hydroxylation of deoxycorticosterone. Incubation of the preparation (25 mg dissolved in 2.0 ml of a 0.154 M solution of KCl) in the presence of 1.0 ml of Tris-MgCl₂ buffer medium, 0.57 µmole of deoxycorticosterone, and 3 µmoles of NADPH results in the formation of 0.09 µmole of corticosterone after 30 minutes of incubation. If the stoichiometry between 11β-hydroxylation and NADPH oxidation is indeed one mole of hydroxylated steroid formed for each mole of NADPH oxidized, then the total amount of NADPH that would be oxidized in the 11β-hydroxylation of deoxycorticosterone to corticosterone would be 0.09 µmole. Thus only a slight increase in the rate of NADPH oxidation would be achieved during the course of 11β-hydroxylation. Such an increase may not be measurable by the experimental methods employed (Fig. 12). These results suggest that the substantial increase in the rate of NADPH oxidation produced by addition of deoxycorticosterone to the acetone powder preparation (Fig. 4) is not entirely a result of 11β-hydroxylation of the steroid substrate.

The presence of cytochrome P-450 in the 11β-hydroxylase prepared by the method of Sharma et al. (33) has been reported
The carbon monoxide difference spectrum of this enzyme system prepared for the present studies was therefore determined. This spectrum, illustrated in Figure 13, is obtained after reduction of the enzyme preparation with NADPH. The intense absorption band with a maximum at 450 mµ is the distinct feature of the spectrum and indicates the presence of cytochrome P-450. In contrast to the acetone powder preparation (Figs. 8 and 10b) the absorption band at 420 mµ is only a minor component of the carbon monoxide difference spectrum of the sonicated enzyme preparation. The results of these experiments (Figs. 12 and 13) indicate that the oxidation of NADPH is accompanied by the reduction of cytochrome P-450, because only the reduced form of this hemoprotein will bind carbon monoxide. Cytochrome P-450 is extremely autoxidizable (129). The cytochrome P-450 reduced during the oxidation of NADPH would be rapidly reoxidized. Therefore the small amounts of cytochrome P-450 present in the sonicated enzyme preparation (5.4 x 10⁻⁵ µmole/mg of powder) may function catalytically to bring about the extensive oxidation of NADPH that is observed (Fig. 12).

A comparison of the 11β-hydroxylase activity and cytochrome P-450 content of acetone powder preparation and the lyophilized sonicate preparation are shown in Table V. The lyophilized sonicate preparation has approximately twice the 11β-hydroxylase activity per milligram of enzyme powder as the acetone powder preparation under the experimental conditions employed. In addition the sonicated preparation contains five times the amount of cytochrome P-450 as the
Fig. 13. NADPH reduced carbon monoxide difference spectrum of an enzyme preparation obtained by ultracentrifugation of sonicated adrenal mitochondria.

Each cuvette contained: 0.05 ml of propylene glycol; 1.0 ml of Tris-MgCl₂ buffer; 1.95 ml of a 0.154 M solution of KCl; 25 mg of lyophilized enzyme preparation; and NADPH (1.1 μmoles) dissolved in 0.1 ml of Tris-MgCl₂ buffer. After 5 minutes of incubation, carbon monoxide was bubbled through the sample cuvette reaction mixture for 60 seconds and the difference spectrum was recorded.
TABLE V

Comparison of the 11β-hydroxylase activity and cytochrome P-450 content in the enzyme systems prepared by extraction of adrenal mitochondrial acetone powder and by ultracentrifugation of sonicated adrenal mitochondria

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>11β-hydroxylase activitya</th>
<th>Cytochrome P-450 contentb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per mg enzyme powder (x10^5)</td>
<td>Per mg protein (x10^5)</td>
</tr>
<tr>
<td>Mitochondrial acetone powder</td>
<td>4.9 ~ 22</td>
<td>0.98</td>
</tr>
<tr>
<td>Lyophilized powder of sonicated mitochondria</td>
<td>9.0 ~ 38</td>
<td>5.4</td>
</tr>
</tbody>
</table>

a 11β-hydroxylase activity = μmole corticosterone formed/min/mg of enzyme powder or protein.

b Cytochrome P-450 content = μmole/mg of enzyme powder or protein.

11β-hydroxylase activity was determined from incubations carried out as described in Fig. 6. Cytochrome P-450 content of the mitochondrial acetone powder preparation was estimated from the data shown in Fig. 10b. Cytochrome P-450 content of the sonicated mitochondrial preparation was determined as shown in Fig. 13 except that sodium dithionite was employed instead of NADPH to reduce the hemoproteins.
acetone powder. Similar results are obtained when 11β-hydroxylase activity and cytochrome P-450 content are expressed on the basis of protein content (Table V). Cytochrome P-450 was estimated using the data of Omura and Sato (89) who determined an extinction coefficient for cytochrome P-450 of 91 cm⁻¹ mM⁻¹ on a protoheme basis, utilizing the change in optical density between 450 and 490 mμ in the carbon monoxide difference spectrum. Although there is a five-fold difference in the cytochrome P-450 content of the enzyme systems prepared by the two different methods, there is only a two-fold difference in the overall 11β-hydroxylase activity. Thus it would seem that the cytochrome P-450 content is not the rate-limiting factor in the hydroxylation reaction.

(ii) Effect of acetone on the sonicated enzyme preparation.

There have been various reports on the decomposition of cytochrome P-450 by treatment with organic solvents (81, 90, 93). Therefore it is likely that the relatively low recovery of this cytochrome from the acetone powder of adrenal mitochondria is a direct result of the methods of preparation of the acetone powder. The effect of acetone on the cytochrome P-450 content of 11β-hydroxylase obtained from sonicated adrenal mitochondria was examined.

Extraction of the lyophilized enzyme preparation with 100% acetone (-20°) produces no alteration in the cytochrome P-450 content (Fig. 14). However, when the preparation is subjected to the same treatment with acetone-water (9:1)
Fig. 14. Effect of extraction with acetone or acetone-water (9:1 v/v) on the cytochrome P-450 content of the sonicated adrenal mitochondria enzyme preparation.

The lyophilized enzyme preparation (200 mg) was extracted with acetone (2 x 10 ml) or acetone-water, 9:1 v/v (2 x 10 ml) and the acetone-insoluble material was dried in vacuo. The cytochrome P-450 content of 25 mg of the extracted preparations was determined as described in Fig. 13. Sodium dithionite was used instead of NADPH to reduce the hemoproteins. Unextracted enzyme preparation (-----); acetone extracted enzyme preparation (-----); acetone-water extracted enzyme preparation (-----).
there is a decrease in cytochrome P-450 content and a corresponding increase in cytochrome P-420. In addition the absorption maximum of cytochrome P-420 is shifted slightly to 418 μm. Thus the acetone treatment causes some alteration of the heme environment of cytochrome P-420 and may explain the fact that the carbon monoxide difference spectrum of cytochrome P-420 isolated from the acetone powder of adrenal mitochondria (Fig. 10b) has absorption maxima at slightly shorter wavelengths than those reported for rabbit liver microsomal cytochrome P-420 by Omura and Sato (88,89). The conversion of cytochrome P-450 to cytochrome P-420 by treatment with acetone-water (9:1) suggests that the phospholipid moiety of cytochrome P-450 is being removed. Acetone-water (9:1) has been shown to remove at least 85% of the phospholipid from mitochondria (109,130). Thus the low cytochrome P-450 content and 11β-hydroxylase activity of the enzyme system obtained from the mitochondrial acetone powder may be attributed to the preparation of the acetone powder.

(e) Studies on Cytochrome P-450 in the Adrenal Mitochondrial Acetone Powder Preparation

Cytochrome P-450 is particulate in nature and possesses unique spectral properties. It is easily converted by a variety of agents to cytochrome P-420 (88-94). In addition, cytochrome P-420 has been found by Omura and Sato (88,89) to be extremely labile under aerobic conditions. Studies on the stability of cytochromes P-450 and P-420 under our experi-
mental conditions were therefore undertaken.

(i) Effect of incubation time and temperature on cytochromes P-450 and P-420.

The effect of incubation temperature on cytochromes P-450 and P-420 is shown in Figure 15. Each reaction mixture was incubated at the indicated temperature for ten minutes with continuous shaking. Solid ammonium sulfate was then added and the fraction containing cytochromes P-450 and P-420 was isolated. The data in Figure 15 indicate that at temperatures below 31°C cytochrome P-450 is relatively stable, the amount recovered after incubation at 31°C being only slightly less than that recovered after incubation at 3°C. A control incubation, to which ammonium sulfate was added prior to incubation at 3°C and in which the aerobic shaking step was omitted, was performed. The amount of cytochrome P-450 recovered from this incubation is almost identical to that of a reaction mixture incubated at this temperature with shaking for 8 minutes. As the incubation temperature is increased from 37°C to 45°C, there is a sharp decrease in the amount of cytochrome P-450 recovered, indicating the extreme lability of this hemoprotein at elevated temperatures.

The effect of incubation temperature on cytochrome P-420 is also shown in Figure 15. The amount of cytochrome P-420 recovered also tends to decrease with increasing incubation temperature. Cytochrome P-420 levels are difficult to interpret
Fig. 15. Effect of incubation temperature on the recovery of cytochromes P-450 and P-420 from the adrenal mitochondrial acetone powder enzyme preparation.

Each reaction flask contained: 0.15 ml of propylene glycol; 2.40 ml of Tris-MgCl$_2$ buffer; and 3.9 ml of enzyme preparation. After 10 minutes of incubation, solid ammonium sulfate was added to give 20% saturation and cytochromes P-450 and P-420 were isolated and measured as described in the methods.

Cytochrome P-450 (O); cytochrome P-420 (●).
however, as the amount measured under the experimental conditions is the net result of the rate of formation of cytochrome P-420 from cytochrome P-450 and the rate of breakdown of cytochrome P-420 itself. That cytochrome P-420 is temperature-labile is evident from the data obtained at 45° C. At this temperature the cytochrome P-450 level has decreased sharply. If cytochrome P-420 is stable at this temperature one would expect an increase in the cytochrome P-420 level resulting from its formation from cytochrome P-450. This is not observed however, the cytochrome P-420 content is also diminished at this temperature when compared to the cytochrome P-420 content at lower incubation temperatures.

The cytochrome P-450 content of a reaction mixture incubated at 37° C as a function of incubation time is shown in Figure 16. There is progressive decrease in the cytochrome P-450 content with increasing incubation time. The decrease in cytochrome P-450 is accompanied by a corresponding increase in cytochrome P-420, illustrating the precursor-product relationship of the two cytochromes. Similar results on cytochrome P-450 breakdown in rabbit liver microsomes have been reported by Ichikawa and Yamano (94). They measured the cytochrome P-450 and P-420 content of microsomes incubated aerobically at 16°C, pH 7.0 over a number of days and observed that the spontaneous decomposition of cytochrome P-450 gave a first-order curve under these conditions. Furthermore the breakdown of cytochrome P-450 was not accompanied by a corresponding increase in cytochrome P-420 indicating that
Fig. 16. Conversion of cytochrome P-450 to cytochrome P-420 as a function of incubation time.

Incubations were carried out as described in Fig. 15. Solid ammonium sulfate to give 20% saturation was added after 0, 4, 8, or 12 minutes of incubation and cytochromes P-450 and P-420 were isolated and measured as described in the methods. Cytochrome P-450 (O); cytochrome P-420 (•).
this hemoprotein was also labile. The rate of breakdown of cytochrome P-450 isolated from the mitochondrial acetone powder (Fig. 16) is much more rapid than the rate of breakdown of cytochrome P-450 of liver microsomes observed by Ichikawa and Yamano. The greater rate of cytochrome P-450 decomposition in the enzyme system from mitochondrial acetone powder could be due to the higher incubation temperature (37° C) used in these studies.

The environment of the cytochrome P-450 in its particulate state may influence the stability of this hemoprotein. Examination of the stability of cytochrome P-450 in the enzyme system prepared by sonication of adrenal mitochondria indicates that this cytochrome is stable under the same experimental conditions that result in a 64% loss of the cytochrome P-450 of the acetone powder preparation. Therefore preparation of the 11β-hydroxylase by acetone treatment of beef adrenal mitochondria results in a particulate cytochrome P-450 that is much more unstable than that obtained by ultrasonic fragmentation of adrenal mitochondria. This instability may be a result of removal of much of the mitochondrial phospholipid by acetone treatment.

The influence of NADPH on the decomposition of cytochrome P-450 was examined and the results are shown in Table VI. The presence of NADPH in the reaction mixture throughout the incubation period does not significantly affect the rate of conversion of cytochrome P-450 to cytochrome P-420. Nishibayashi and Sato (84) have reported that under aerobic steady
### TABLE VI

Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation incubated in the presence or absence of NADPH

<table>
<thead>
<tr>
<th>Incubation time (minutes)</th>
<th>Δ Absorbance 450-500 μm</th>
<th>420-500 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No NADPH</td>
<td>Plus NADPH</td>
</tr>
<tr>
<td>0</td>
<td>0.026</td>
<td>0.028</td>
</tr>
<tr>
<td>4</td>
<td>0.017</td>
<td>0.020</td>
</tr>
<tr>
<td>8</td>
<td>0.011</td>
<td>0.012</td>
</tr>
<tr>
<td>12</td>
<td>0.009</td>
<td>0.010</td>
</tr>
</tbody>
</table>

Each reaction flask contained: 0.15 ml of propylene glycol; 2.25 ml of Tris-MgCl₂ buffer; 3.9 ml of enzyme preparation; NADPH (3.3 μmoles) dissolved in 0.15 ml of Tris-MgCl₂ buffer when appropriate. After 0, 4, 8, or 12 minutes of incubation, solid ammonium sulfate was added to give 20% saturation and cytochromes P-450 and P-420 were isolated and measured as described in the methods.
state conditions approximately 30% of the cytochrome P-450 of liver microsomes was reduced by NADPH. Cammer and Estabrook (85) have observed that the cytochrome P-450 of beef adrenal mitochondria is also largely in the oxidized form during the aerobic steady state of deoxycorticosterone hydroxylation. Therefore the lack of any demonstrable effect of NADPH on the stability of cytochrome P-450 (Table VI) is not an unexpected finding. Cytochrome P-450 is essentially in the same form (i.e. oxidized form) in the presence of NADPH as in its absence. Omura and Sato (88,89) have reported that cytochrome P-420 is more unstable in the reduced form than in the oxidized form under aerobic conditions. Similarly Imai and Sato (90) have observed that the cytochrome P-450 of liver microsomes was more susceptible to decomposition by the action of neutral salts when in its reduced state. The present results with cytochrome P-450 indicate that the oxidized form of cytochrome P-450 is also extremely unstable (Fig. 16) and that NADPH under aerobic steady state conditions does not significantly alter this lability (Table VI).

(ii) Effect of steroid substrates on cytochromes P-450 and P-420.

The ability of substrates to stabilize enzymes has been well documented (131-133). Therefore it seemed of importance to examine the effects of both substrates and products of the 11β-hydroxylase on cytochrome P-450.
Incubation of the enzyme preparation at 37° C in the presence of substrate deoxycorticosterone results in an increased recovery of cytochrome P-450 (Fig. 17). This increase is proportional to the amount of deoxycorticosterone present and is maximal at deoxycorticosterone concentrations in the region of the $K_m$ for 11β-hydroxylation (5.5 μmole/l). These results suggest that deoxycorticosterone is binding to cytochrome P-450 and protecting it from degradation perhaps by shielding some labile group or by producing some conformational change in the cytochrome. There is now substantial spectrophotometric evidence that steroid substrates do bind to cytochrome P-450 (95-98). The protective effect of deoxycorticosterone on cytochrome P-450 illustrated in Figure 17 is indicative of steroid binding to cytochrome P-450.

Other steroids were also examined for their effect on cytochrome P-450. Corticosterone, the product of 11β-hydroxylation of deoxycorticosterone, has no protective effect at all concentrations tested (Fig. 17). Androstenedione, which has been shown by Sharma et al. (41) to be a competitive inhibitor of 11β-hydroxylation of deoxycorticosterone, protects cytochrome P-450 in a manner analogous to that of deoxycorticosterone (Fig. 18). The presence of an 11-oxo group in adrenosterone completely eliminates this protective effect (Fig. 18). The protective effects of androstenedione and deoxycorticosterone on cytochrome P-450, and the competitive inhibition of 11β-hydroxylation of deoxycorticosterone by androstenedione demonstrated by Sharma et al. (41) suggest
Fig. 17. Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of deoxycorticosterone or corticosterone.

Each reaction flask contained: steroid dissolved in 0.15 ml of propylene glycol to give the indicated final concentration; 2.1 ml of Tris-MgCl₂ buffer; 3.9 ml of enzyme preparation; and 0.15 ml of a dilute alcoholic KOH solution. Each reaction flask was pre-incubated for 8 minutes. NADPH (3.3 µmoles) dissolved in 0.15 ml of Tris-MgCl₂ buffer was added. After 90 seconds of incubation, solid ammonium sulfate was added to give 20% saturation and cytochrome P-450 then was isolated and measured as described in the methods.

Steroid added: deoxycorticosterone (□); corticosterone (□).
Fig. 18. Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of androstenedione or adrenosterone.

Incubations were carried out as described in Fig. 17. Steroid added: androstenedione (o); adrenosterone (o).
that these steroids bind at the same site on the cytochrome P-450 molecule.

Imai and Sato (90) have found, during studies on substrate interaction with cytochrome P-450 in rabbit liver microsomes, that high concentrations of the substrate aniline resulted in some conversion of cytochrome P-450 to cytochrome P-420. The effect of high substrate deoxycorticosterone concentrations under our experimental conditions is shown in Figure 19. At the highest concentration tested, 400 μmoles/l, deoxycorticosterone still provides the same degree of protection of cytochrome P-450 as at lower concentrations and the substrate-produced decomposition of cytochrome P-450 noted by Imai and Sato (90) is not apparent in the deoxycorticosterone-cytochrome P-450 system employed in our experiments.

In an attempt to establish the manner in which deoxycorticosterone protects cytochrome P-450, the rates of decomposition of cytochrome P-450 in the absence and in the presence of deoxycorticosterone substrate were compared (Fig. 20). In the absence of deoxycorticosterone there is a rapid initial breakdown of cytochrome P-450 during the first four minutes of incubation. In the presence of deoxycorticosterone this rate of decomposition is reduced by approximately one-half. Therefore the elevated cytochrome P-450 levels produced by deoxycorticosterone in Figure 17 are the result of a diminution in the rate of decomposition of this cytochrome. The binding of deoxycorticosterone to cytochrome P-450 retards the rate of oxidative decomposition
Fig. 19. Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of high concentrations of deoxycorticosterone.

Incubations were carried out as described in Fig. 17. Cytochrome P-450 (o); cytochrome P-420 (o).
Fig. 20. Effect of deoxycorticosterone on the rate of cytochrome P-450 disappearance.

Each reaction flask contained: 0.15 ml of propylene glycol containing deoxycorticosterone (38.8 μmoles/l, final concentration) or 0.15 ml of propylene glycol (control); 2.25 ml of Tris-MgCl₂ buffer; 3.9 ml of enzyme preparation; and 0.15 ml of a dilute alcoholic KOH solution. After incubation for 0, 4, 8, or 12 minutes, solid ammonium sulfate was added to each flask to give 20% saturation and cytochromes P-450 and P-420 were isolated and measured as described in the methods.

Cytochrome P-450: control (circles), plus deoxycorticosterone (O). Cytochrome P-420: control (circles), plus deoxycorticosterone (O).
of this hemoprotein either by protecting some labile group of the cytochrome, or by inducing some conformational change which renders the cytochrome more stable.

Measurement of cytochrome P-420 levels in the presence of deoxycorticosterone (38.8 μmoles/l) indicates that no substrate protection is afforded this hemoprotein at this substrate concentration (Fig. 20). In the experiments with extremely high concentrations of deoxycorticosterone (up to 400 μmoles/l) some protection of cytochrome P-420 is observed, the recovery of cytochrome P-420 being about 75% higher in the presence of deoxycorticosterone (400 μmoles/l) than in its absence (Fig. 19). This protection of cytochrome P-420 suggests that deoxycorticosterone can bind to this hemoprotein but the substrate affinity for cytochrome P-420 is much lower than that for cytochrome P-450. Imai and Sato (90,98) have observed the binding of the substrate aniline to a partially purified cytochrome P-420 from rabbit liver microsomes. The substrate concentration required for half-maximal binding to cytochrome P-420 was approximately 50 times higher than that observed for the binding of aniline to cytochrome P-450. These observations of Imai and Sato concur with those of the present experiments. Deoxycorticosterone affords protection of cytochrome P-420 only at concentrations much higher than those which maximally stabilize cytochrome P-450.

DISCUSSION

In early studies on enzymatic 11β-hydroxylation much
effort was expended by several groups of investigators on defining the cofactor requirements of the 11β-hydroxylase. These studies indicated that the reaction was an aerobic one requiring, or stimulated by, one or more of NAD, NADPH, ATP, Mg$^{++}$ as well as fumarate or other intermediates of the Krebs citric acid cycle (22-25). Clarification of the function of these individual components was provided independently by Grant (31) and by Sweat and Lipscomb (8) who demonstrated that the actual cofactor for 11β-hydroxylation was NADPH and that the other compounds were in fact concerned with the generation of NADPH.

The absolute requirement of the 11β-hydroxylase extracted from a bovine adrenal mitochondrial acetone powder for NADPH has been demonstrated in the present studies (Table I). Indeed significant conversion of deoxycorticosterone to corticosterone required a high molar ratio of NADPH to steroid substrate. Spectrophotometric examination of NADPH oxidation by the enzyme preparation revealed that extensive oxidation of this cofactor occurred even in the absence of steroid substrate. The oxidation of NADPH was found to be insensitive to potassium cyanide, Antimycin A, and amytal and was therefore not proceeding via the classical electron transport chain. In addition, spectrophotometric analysis of the enzyme preparation indicated that the only cytochrome of the classical electron transport chain enzymatically reduced by NADPH was cytochrome c (Fig. 7).
Because the enzyme preparation employed in these studies is a crude extract of a mitochondrial acetone powder, oxidation of NADPH catalyzed by enzymes not involved in 11β-hydroxylation is a distinct possibility. The NADPH-reduced minus oxidized difference spectrum of the enzyme preparation (Fig. 7) exhibited an absorption spectrum similar to cytochrome c. This would indicate the presence of an NADPH-cytochrome c reductase in this enzyme preparation which could in part at least account for the observed oxidation of NADPH in the absence of steroid substrate. Cytochrome P-420 was also shown to be a component of this enzyme preparation. This cytochrome is not a normal constituent of adrenal mitochondria but is produced by the breakdown of cytochrome P-450. This decomposition can be brought about by detergents, (88,89), phospholipase (88,89), and organic solvents (81,90,93) as well as other agents. In the present studies it was found that acetone-water (9:1) could cause the conversion of cytochrome P-450 to cytochrome P-420 (Fig. 14) and the cytochrome P-420 present in the enzyme preparation was produced during the formation of the mitochondrial acetone powder. The breakdown of cytochrome P-450 by acetone-water (9:1), as well as by detergents and phospholipase is accompanied by the removal of phospholipid. Cytochrome P-420 can be reduced enzymatically by NADPH (59,60,88) but does not function in 11β-hydroxylation. The presence of this cytochrome in the enzyme system extracted from the acetone powder of bovine adrenal mitochondria could be responsible for much of the oxidation of NADPH, necessitating
the addition of excessive amounts of this cofactor to demonstrate quantitative 11β-hydroxylation of added steroid substrate.

NADPH-supported 11β-hydroxylation is insensitive to the electron transport inhibitors antimycin A and amyntal but is inhibited by dicumarol (Table IV). This inhibitor is known to uncouple oxidative phosphorylation in the classical electron transport chain (122-124). However its ability to inhibit 11β-hydroxylation under the experimental conditions employed in these studies is unusual, for it has been demonstrated that the classical electron transport chain is not involved in NADPH-supported 11β-hydroxylation. Inhibition of 11β-hydroxylation supported by the Krebs cycle intermediate, succinate, by electron transport inhibitors and uncoupling agents has been observed (24,39,115,117,126,134). Within the adrenal mitochondrion an energy-linked reduction of NAD by succinate can occur. A pyridine nucleotide transhydrogenase can then bring about the reduction of NADP. That these two reactions are energy-linked explains the sensitivity of succinate-supported 11β-hydroxylation to electron transport inhibitors and uncoupling agents. It is believed that in adrenocortical mitochondria there is an interaction between the classical respiratory chain and the 11β-hydroxylase system (39,115) with the result that oxidation of substrates by the conventional respiratory chain can ultimately lead to transfer of electrons to the 11β-hydroxylase pathway. However, NADPH-supported 11β-hydroxylation is not energy-linked.
Therefore dicumarol must be inhibiting this reaction by a mechanism distinct from its effect on oxidative phosphorylation. A detailed study on the mechanism of this inhibition will be discussed in a later part of this thesis.

Cytochrome P-450 serves as the terminal oxidase for the 11β-hydroxylation of deoxycorticosterone (56,57,72) and its presence in adrenal mitochondria has been well established (57,69-71). The 11β-hydroxylase prepared by extraction of an adrenal mitochondrial acetone powder with a potassium chloride solution contains this cytochrome (Fig. 10b) although in lesser amounts than the 11β-hydroxylase prepared by different methods (Table V). This hemoprotein can only be detected after fractionation of the enzyme preparation with ammonium sulfate. The carbon monoxide difference spectrum of the fraction containing cytochrome P-450 also exhibits an absorption maximum at 420 μm which may be due to contributions by cytochrome P-420 and hemoglobin.

The cytochrome P-450 isolated from the acetone powder of adrenal mitochondria is extremely labile and undergoes spontaneous decomposition at temperatures higher than 30° C to cytochrome P-420 (Figs. 15 and 16). A similar breakdown of cytochrome P-450 under aerobic conditions has been observed by Omura and Sato (89), Ichii et al. (80), and Ichikawa and Yamano (94). Cytochrome P-420 is also unstable and is degraded during incubation. The loss of cytochrome P-420 is accompanied by a loss of total protoheme (89). Omura
and Sato (89) observed that both cytochromes P-450 and P-420 were stabilized under anaerobic conditions suggesting that the breakdown of the cytochromes involved interaction with oxygen. Ichikawa and Yamano (94) and Ichii et al. (80) demonstrated that the aerobic decomposition of cytochrome P-450 was prevented in the presence of glycerol. Results of the present experiments indicate that cytochrome P-450 is stabilized in the presence of steroid substrate (Figs. 17-20). This effect appears to be specific for the substrates of the steroid 11β-hydroxylase. The presence of a hydroxyl or oxo function at the C-11 position of the steroid nucleus eliminates the protective effect. The presence of a C-17 sidechain does not appear important, however, as androstenedione is as effective as deoxycorticosterone in protecting cytochrome P-450.

Very little is known about the substrate specificity of cytochrome P-450. More extensive investigations of the type outlined in Figures 17 and 18 could aid in the assignment of the requisite structural aspects of the steroid molecule involved in enzyme binding and specificity of the 11β-hydroxylase.

The protective effect of deoxycorticosterone on cytochrome P-450 could possibly account for the stoichiometry of greater than unity observed for NADPH oxidation and 11β-hydroxylation (Fig. 4). Incubations containing deoxycorticosterone would also contain more cytochrome P-450 than those without deoxycorticosterone. This higher cytochrome P-450 content could
result in a greater rate of NADPH oxidation even though it may not be associated with 11β-hydroxylation.

The mechanism whereby deoxycorticosterone protects cytochrome P-450 must await further studies on the nature of this hemoprotein. The conversion of cytochrome P-450 to cytochrome P-420 results from an alteration of the hydrophobic nature of this hemoprotein. The unusual spectral properties of cytochrome P-450 are believed to be a result of hydrophobic interaction of the heme with hydrophobic areas of the P-450 protein or with lipids present in the cytochrome P-450 complex. Any alteration of this interaction therefore results in the conversion of cytochrome P-450 to cytochrome P-420. As the steroid substrates for 11β-hydroxylation are hydrophobic in nature, it is reasonable to assume that these molecules can penetrate to the reactive area of the cytochrome P-450 complex, i.e., the active site, and maintain the hydrophobic nature of this complex either by protecting some labile group, or by altering the cytochrome to a more stable conformation. As this protective effect is afforded only by the substrate steroids, and not by the products of 11β-hydroxylation (e.g., corticosterone), the substrate binding site would appear to be involved. The inability of corticosterone and adrenosterone to stabilize cytochrome P-450 suggests that the stabilization is not a general effect produced by any hydrophobic compound.

An alternative mechanism for increased levels of cytochrome P-450 in the presence of steroid substrates could be
the reconversion of cytochrome P-420 to cytochrome P-450 induced by the addition of steroid. Ichikawa and Yamano (94) have observed the reconversion of detergent and sulfhydryl reagent-produced cytochrome P-420 to cytochrome P-450 by polyols such as glycerol and ethylene glycol, and by glutathione. They demonstrated reconversion at temperatures as low as 0° C. A similar deoxycorticosterone-induced conversion of cytochrome P-420 to P-450 would explain the higher cytochrome P-450 levels obtained in the presence of deoxycorticosterone in the zero time control incubation (maintained at 4° C) of Figure 20, even though the hemoprotein is relatively stable at this temperature (Fig. 15). The existence of cytochrome P-420 in various forms has been suggested (81). It is possible that the binding of deoxycorticosterone to one of the cytochrome P-420 states can produce a conformational change resulting in a reformation of cytochrome P-450 perhaps due to an increase in hydrophobic interactions in the altered cytochrome P-420 complex. Both of the above postulated mechanisms may be contributing to the increased levels of cytochrome P-450 evident in the presence of steroid substrate.
PART II THE ROLE OF QUINONES IN 11β-HYDROXYLATION

The evidence presented in Part I of this thesis suggested that the inhibition of 11β-hydroxylation by dicumarol did not result from the uncoupling of oxidative phosphorylation by this inhibitor. Numerous reports have been published describing dicumarol-sensitive systems in animal tissues which can utilize quinones as electron acceptors and in which quinones can mediate the aerobic oxidation of NADPH or NADH by molecular oxygen (62,109,135-141). The quinone, Coenzyme Q₁₀, is known to be present in mammalian mitochondria (142). It therefore seemed possible that the dicumarol inhibition of 11β-hydroxylation demonstrated in our experiments could be explained by inhibition of some reaction involving a quinone (vitamin K or coenzyme Q) compound. This would imply a function for vitamin K or coenzyme Q in 11β-hydroxylation. Fitch and Folkers (143) have reported that coenzyme Q₁₀ has the capacity to stabilize biological membranes. Thus there is a possibility that coenzyme Q₁₀ is associated with cytochrome P-450 which is also membrane-bound. Alternatively a quinone may be functioning in the electron transport sequence of the 11β-hydroxylase pathway. A study of the possible involvement of a vitamin K or coenzyme Q-type compound in 11β-hydroxylation was therefore undertaken.

RESULTS

(a) Effect of Acetone-Extracted Lipid and Menadione (Vitamin K₃) on NADPH Oxidation
During the preparation of the adrenal mitochondrial acetone powder, a majority of the phospholipids and quinones are extracted from the mitochondria by acetone (109,130,144,145). If these compounds are involved in 11β-hydroxylation and NADPH oxidation then the 11β-hydroxylase prepared from the acetone powder would have a reduced activity compared to a system prepared by an alternate method (Table V). The total acetone-extracted fraction was therefore examined for its effect on NADPH oxidation.

Addition of a sonicated suspension of the acetone-extracted material results in a substantial increase in the rate of NADPH oxidation (Fig. 21). This increased oxidation of NADPH is dependent on the presence of the enzyme preparation as is evidenced by the lack of NADPH oxidation in its absence. Acetone extracts from different adrenal mitochondrial acetone powder preparations were examined, and although the absolute stimulation of NADPH oxidation varied from extract to extract, similar results to those shown in Figure 21 were generally observed. Kamin et al. (62) have reported that the NADPH oxidase activity of a purified NADPH-cytochrome c reductase from liver microsomes could be markedly stimulated by lipid extracts derived from liver and adrenal cortex although they found the stimulatory effect to be extremely variable. The purified enzyme also exhibited NADPH-menadione reductase activity. NADPH oxidation by the enzyme preparation employed in the present studies is also stimulated by menadione (2-methyl-1,4-naphthoquinone) (Fig. 22). Thus NADPH oxidation
Fig. 21. Effect of the acetone-extracted mitochondrial lipid fraction on NADPH oxidation.

Each reaction flask contained: 1.0 ml of Tris-MgCl\(_2\) buffer; 2 ml of enzyme preparation; 0.02 ml of a sonicated lipid suspension containing 150 mg lipid/ml (the control reaction flask received 0.02 ml of Tris-MgCl\(_2\) buffer). 0.9 \(\mu\)mole of NADPH in 0.1 ml of Tris-MgCl\(_2\) buffer was added to each reaction flask at zero time.

Control (○); plus lipid, 0.96 mg/ml, (○); lipid control, 2 ml of a 0.154 M KCl solution instead of enzyme preparation added to a reaction mixture containing lipid, 0.96 mg/ml (■).
Fig. 22. Effect of menadione on NADPH oxidation.

Each reaction flask contained: 1.0 ml of Tris-MgCl$_2$ buffer; 2.0 ml of enzyme preparation; 0.00, 0.05, or 0.10 μmole of menadione dissolved in 0.1 ml of ethanol. NADPH (0.7 μmole) dissolved in 0.1 ml of Tris-MgCl$_2$ buffer was added to each reaction flask at zero time.

Control (○); plus menadione, 3.13 (■) or 15.7 (▲) μmoles/1 final concentration; menadione control, 2 ml of a 0.154 M KCl solution instead of enzyme preparation was added to a reaction mixture containing menadione, 31.3 μmoles/1, final concentration (▲).
by the acetone powder enzyme is affected by menadione and by lipid extracts in a manner similar to the microsomal NADPH-cytochrome c reductase described by Kamin et al. (62). The presence of menadione (16 μmoles/l, final concentration) produces a greater than two-fold increase in the initial rate of NADPH oxidation (Fig. 22).

The specificity of the menadione-stimulated NADPH oxidase activity was examined and the results are shown in Figure 23. 1,4-Naphthoquinone produces an extensive stimulation of the rate of NADPH oxidation while 1,4-toluquinone and 1,4-benzoquinone, members of the coenzyme Q series, have only a slight stimulatory effect. Of the two benzoquinones, 1,4-toluquinone is more effective, producing a greater stimulation of NADPH oxidation than 1,4-benzoquinone at one-tenth the concentration of benzoquinone. Therefore the menadione reductase of the mitochondrial acetone powder extract is essentially specific for naphthoquinones.

Ernster et al. (136,137) have shown that the soluble fraction of rat liver cytoplasm contains a dicumarol-sensitive diaphorase catalyzing the reduction of various quinones and dyes with either NADH or NADPH as electron donor. However the microsomal NADPH-cytochrome c reductase described by Sato et al. (146,147) was insensitive to dicumarol, even at high concentrations (0.2 mM) of this compound. The effect of dicumarol on the acetone extract-stimulated and menadione-stimulated NADPH oxidation in the mitochondrial acetone powder enzyme preparation was therefore examined. The initial rate of NADPH oxidation in the presence of acetone-extracted lipid,
Fig. 23. Effect of 1,4-naphthoquinone, 1,4-toluquinone, and 1,4-benzoquinone on NADPH oxidation.

Incubations were carried out as described in Fig. 22. 1,4-naphthoquinone (0.1 µmole), 1,4-toluquinone (0.1 µmole), or 1,4-benzoquinone (1.0 µmole) dissolved in 0.1 ml ethanol was added instead of menadione.

Control (o); plus 1,4-naphthoquinone (o); plus 1,4-toluquinone (△); plus 1,4-benzoquinone (□).
menadione, and dicumarol are shown in Table VII. Addition of the acetone extract (3.1 mg/ml final concentration) alone produces a four-fold increase in the rate of NADPH oxidation. Menadione (31 μmoles/l) produces a two-fold increase in NADPH oxidation, while dicumarol produces only a slight stimulation. When dicumarol is added to the reaction mixture containing acetone-extracted lipid there is a two-fold increase in the rate of NADPH oxidation over that produced by the addition of the acetone extract alone. This stimulation is even more pronounced when dicumarol is added in the presence of menadione, resulting in a six-fold increase in the rate of NADPH oxidation over that produced by menadione alone. Thus the oxidase activity of the mitochondrial acetone powder enzyme preparation differs from both the dicumarol-inhibited diaphorase described by Ernster et al. (136,137) and the dicumarol-insensitive NADPH-naphthoquinone reductase (NADPH-cytochrome c reductase) described by Sato et al. (146,147). This dicumarol-produced stimulation of NADPH oxidation in the presence of menadione (Table VII) is extremely sensitive to dicumarol and is observed at dicumarol concentrations as low as 1.6 x 10^{-8} M. The enhancing effect of dicumarol on both the menadione-stimulated and acetone-extracted lipid-stimulated oxidation of NADPH suggests the possibility that factors in the lipid extract responsible for this stimulation may be compounds analogous to menadione.

(b) Effect of Acetone-Extracted Lipid and Menadione on 11β-Hydroxylation

Having established the effects of the acetone-extracted
### TABLE VII

Effect of mitochondrial acetone-extracted lipid, menadione, and dicumarol on the initial rate of NADPH oxidation

<table>
<thead>
<tr>
<th>Additions</th>
<th>Initial rate of NADPH oxidation Δ Absorbance (340 μm) per minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.021</td>
</tr>
<tr>
<td>Lipid (3.1 mg/ml)</td>
<td>0.084</td>
</tr>
<tr>
<td>Menadione (31 μmoles/l)</td>
<td>0.049</td>
</tr>
<tr>
<td>Dicumarol (155 μmoles/l)</td>
<td>0.030</td>
</tr>
<tr>
<td>Lipid plus dicumarol</td>
<td>0.164</td>
</tr>
<tr>
<td>Menadione plus dicumarol</td>
<td>0.290</td>
</tr>
</tbody>
</table>

Appropriate reaction flasks contained: 1.0 ml of Tris-MgCl₂ buffer; 2.0 ml of enzyme preparation; lipid (10 mg) suspended in 0.1 ml of Tris-MgCl₂ buffer; menadione (0.1 μmole) dissolved in 0.02 ml of ethanol, dicumarol (0.5 μmole) dissolved in 0.05 ml of a dilute alcoholic KOH solution. The appropriate solvent was added to reaction flasks not receiving lipid, menadione, or dicumarol. NADPH (0.8 μmole) dissolved in 0.05 ml of Tris-MgCl₂ buffer was added at zero time.
lipid and menadione on NADPH oxidation, it was deemed important to determine whether or not similar effects on \(11\beta\)-hydroxylation were exerted by these factors.

The acetone-extracted lipid fraction and menadione were examined for their effects on the NADPH oxidation associated with the hydroxylation of substrate deoxycorticosterone. The effect of the acetone-extracted lipid fraction is shown in Figure 24. The addition of substrate deoxycorticosterone (186 \(\mu\)moles/l, final concentration) increases the initial rate of NADPH oxidation from 10 \(\mu\)moles/min to 15 \(\mu\)moles/min. Addition of acetone-extracted lipid increases the initial rate of NADPH oxidation from 10 \(\mu\)moles/min to 45 \(\mu\)moles/min. Addition of deoxycorticosterone in the presence of lipid results in a further stimulation of NADPH oxidation, to 58 \(\mu\)moles/min. Thus the increase in the rate of NADPH oxidation produced by deoxycorticosterone in the presence of the acetone-extract (13 \(\mu\)moles/min) is greater than that produced by the deoxycorticosterone in the absence of this lipid (5 \(\mu\)moles/min) and suggests that the lipid extract is increasing the rate of \(11\beta\)-hydroxylation. NADPH oxidation in the presence of dicumarol is not affected by the addition of substrate deoxycorticosterone (Fig. 24). Therefore dicumarol inhibits the increase in NADPH oxidation that is normally produced by the addition of deoxycorticosterone (Figs. 4 and 24), a finding in agreement with the established inhibitory action of dicumarol on \(11\beta\)-hydroxylation. This inhibition of deoxycorticosterone-associated NADPH oxidation is not overcome by the addition of the acetone-extracted lipid fraction (Fig.
Fig. 24. Effect of deoxycorticosterone, mitochondrial acetone-extracted lipid, and dicumarol on NADPH oxidation.

Appropriate reaction flasks contained: deoxycorticosterone (186 μmoles/l, final concentration) dissolved in 0.05 ml of propylene glycol; 1.0 ml of Tris-MgCl₂ buffer; 2.0 ml of enzyme preparation; acetone-extracted lipid (10 mg) suspended in 0.1 ml of Tris-MgCl₂ buffer; and dicumarol (0.5 μmole) dissolved in 0.05 ml of a dilute alcoholic KOH solution. The appropriate solvent was added to reaction flasks not receiving deoxycorticosterone, lipid, or dicumarol. NADPH (0.8 μmole) dissolved in 0.05 ml of Tris-MgCl₂ buffer was added at zero time.

Control (○—○); plus deoxycorticosterone (○—○); plus dicumarol (○—○); plus dicumarol and deoxycorticosterone (○—○); plus lipid (△—△); plus lipid and deoxycorticosterone (△—△); plus lipid and dicumarol (○—○); plus lipid, dicumarol and deoxycorticosterone (○—○).
24). The rapid oxidation of NADPH in the presence of both dicumarol and the acetone-extracted lipid is not altered upon addition of deoxycorticosterone.

The effect of menadione on NADPH oxidation associated with 11β-hydroxylation is shown in Figure 25. In this instance the increase in NADPH oxidation produced by the addition of deoxycorticosterone in the presence of menadione is no greater than that resulting from the addition of deoxycorticosterone in the absence of menadione. Therefore it appears that menadione is not increasing the rate of 11β-hydroxylation. The extensive stimulation of NADPH oxidation produced by the simultaneous addition of menadione and dicumarol occurs in both the absence and presence of steroid substrate. While the effects of both the acetone-extracted lipid fraction and menadione on "background" NADPH oxidation in both the absence and presence of dicumarol are similar, the acetone-extracted lipid fraction possesses a factor (or factors) that increases the rate of 11β-hydroxylation. A more critical examination of the effects of menadione and the acetone-extracted lipid material on 11β-hydroxylation was accomplished by direct measurement of corticosterone formation. The results of these studies are shown in Figure 26. In the presence of the acetone-extracted lipid fraction there is a stimulation of the rate of corticosterone formation, resulting in a 27% increase in the amount of corticosterone formed after 8 minutes of incubation. This finding is in agreement with the stimulation of deoxycorticosterone-associated NADPH oxidation shown in Figure 24. Similar re-
Fig. 25. Effect of deoxycorticosterone, menadione and dicumarol on NADPH oxidation.

Incubations were carried out as described in Fig. 24. Menadione (0.1 µmole) dissolved in 0.02 ml of ethanol was added instead of the mitochondrial acetone-extracted lipid.

Control (○); plus deoxycorticosterone (●); plus menadione (○); plus menadione and deoxycorticosterone (□); plus menadione and dicumarol (▲); plus menadione, deoxycorticosterone, and dicumarol (●).
Fig. 26. Effect of mitochondrial acetone-extracted lipid, menadione, and dicumarol on the 11β-hydroxylation of deoxycorticosterone.

Incubations were carried out as described in Figs. 24 and 25. Deoxycorticosterone-1,2-³H (specific activity 1.07 x 10⁶ cpm/µmole) was added dissolved in 0.05 ml of propylene glycol to each reaction flask to give a final concentration of 197 µmoles/l. NADPH (2.2 µmoles) dissolved in 0.08 ml of Tris-MgCl₂ buffer was added at zero time. After 8, 16, and 24 minutes of incubation, a sample (1.0 ml) was removed from each reaction flask and pipetted into ethyl acetate (2 ml) to terminate the reaction.

Control (○); plus lipid (△); plus menadione (□); plus dicumarol (◊); plus lipid and dicumarol (▲); plus menadione and dicumarol (●).
suits have been reported by Imai and Sato (148,149) who found that the aromatic hydroxylation of aniline by an acetone powder of rabbit-liver microsomes was dependent upon a lipid fraction extracted from these microsomes by treatment with an acetone-methanol-ether mixture. The inhibition of 11β-hydroxylation by dicumarol (62% after 8 minutes of incubation) is only slightly relieved by the addition of the acetone-extracted lipid fraction.

Menadione produces no increase in corticosterone formation; indeed on prolonged incubation, there is a decrease in the rate of 11β-hydroxylation in the presence of menadione when compared to the control rate (Fig. 26). This decrease in the rate of 11β-hydroxylation is likely due to the increased utilization of NADPH in the presence of menadione (Fig. 25). Thus under conditions of limited availability of NADPH, menadione becomes an inhibitor of 11β-hydroxylation due to competition with the 11β-hydroxylase for NADPH. This is evident from the high degree of inhibition of 11β-hydroxylation obtained upon addition of both menadione and dicumarol (Fig. 26), caused by the extremely rapid utilization of NADPH (Fig. 25).

(c) A Possible Site for Menadione Reduction

In recent studies on the electron-transport system involved in 11β-hydroxylation, Kimura (60) has observed that ferricyanide, menadione, or cytochrome c, as well as the natural acceptor, adrenodoxin, can act as electron acceptors for the oxidation of NADPH by adrenodoxin reductase. Of
the electron acceptors tested by Kimura, ferricyanide was the most effective. Maximal adrenodoxin reductase activity in the presence of menadione and cytochrome c was respectively 41% and 7% of that in the presence of adrenodoxin.

Kimura and Suzuki (59) have reported that cytochrome P-450, cytochrome P-420, and the flavoprotein adrenodoxin reductase of the 11β-hydroxylase extracted from an adrenal mitochondria-acetone powder are all present in a fraction precipitating between 20% and 40% saturation with ammonium sulfate. These findings have been confirmed in the present studies (Figs. 10a and 10b). This ammonium sulfate fraction is essentially devoid of adrenodoxin which precipitates at higher ammonium sulfate concentrations (59). NADPH oxidation by the 20-40% ammonium sulfate fraction, and the effect of menadione upon this oxidation were therefore examined.

NADPH oxidation by the fractions precipitating between 20% and 40%, and 40% and 80% saturation with ammonium sulfate is shown in Table VIII. The 20-40% ammonium sulfate fraction oxidizes NADPH at a slow rate. Determination of the carbon monoxide difference spectrum of this fraction in the presence of NADPH indicates that the slow oxidation of NADPH is accompanied by a small degree of reduction of both cytochrome P-450 (Δ absorbance 450-500 μm) and cytochrome P-420 (Δ absorbance 420-500 μm). Reduction of the total hemoprotein content of the 20-40% ammonium sulfate fraction with dithionite indicates that only a small portion of the total cytochromes present is reduced by NADPH. This slow
TABLE VIII

The relationship between oxidation of NADPH and reduction of cytochromes P-450 and P-420 in the 20-40% and 40-80% ammonium sulfate fractions of the mitochondrial acetone powder enzyme preparation

<table>
<thead>
<tr>
<th>(NH₄)₂SO₄ fraction incubated</th>
<th>NADPH oxidation</th>
<th>NADPH-reduced P-450 &amp; P-420</th>
<th>Dithionite-reduced P-450 &amp; P-420</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δ Absorbance (340 μm) per minute</td>
<td>Δ Absorbance 450-500 μm</td>
<td>Δ Absorbance 420-500 μm</td>
</tr>
<tr>
<td>20-40% (no NADPH added)</td>
<td>0.000</td>
<td>0.000</td>
<td>0.004</td>
</tr>
<tr>
<td>20-40%</td>
<td>0.015</td>
<td>0.008</td>
<td>0.013</td>
</tr>
<tr>
<td>40-80% (0.1 ml)</td>
<td>0.010</td>
<td>0.000</td>
<td>0.12</td>
</tr>
<tr>
<td>40-80% (0.2 ml)</td>
<td>0.022</td>
<td>0.000</td>
<td>0.24</td>
</tr>
<tr>
<td>(20-40%) + 0.1 ml (40-80%)</td>
<td>0.035</td>
<td>0.004</td>
<td>0.12</td>
</tr>
<tr>
<td>(20-40%) + 0.2 ml (40-80%)</td>
<td>0.060</td>
<td>0.006</td>
<td>0.26</td>
</tr>
</tbody>
</table>

The 20-40% and 40-80% ammonium sulfate fractions obtained from 4 ml of the enzyme preparation were each dissolved in a solution made up of Tris-MgCl₂ buffer (0.42 ml) and 0.154 M KCl (0.74 ml). Appropriate cuvettes contained: 0.58 ml of the 20-40% fraction; 0.1 or 0.2 ml of the 40-80% fraction; 0.01 ml of ethanol, and 0.02 ml of propylene glycol. The volume of the reaction mixture was adjusted to 0.81 ml with the Tris-KCl solution. NADPH oxidation was measured by addition of NADPH (0.27 μmole) to the sample cuvette. Reduction of cytochromes P-450 and P-420 was measured (after addition of NADPH (0.27 μmole) or dithionite to both the sample and reference cuvettes) by determining the carbon monoxide difference spectrum of the reaction mixture.
rate of NADPH oxidation and limited reduction of cytochromes P-450 and P-420 are expected results because adrenodoxin, the component of the 11β-hydroxylase pathway which functions in the transfer of electrons from adrenodoxin reductase to cytochrome P-450, is not present in this ammonium sulfate fraction. The small amounts of cytochrome P-450 and cytochrome P-420 that are reduced may indicate the presence of trace amounts of adrenodoxin in this fraction.

The 40-80% ammonium sulfate fraction also catalyzes a slow rate of NADPH oxidation (Table VIII), perhaps via some other flavoprotein that is present in this fraction (Fig. 11a). Recombination of the 20-40% and 40-80% fraction results in an increase in NADPH oxidation, the increased rate being greater than the sum of the NADPH oxidized by both fractions alone. Unfortunately attempts to show that this increased oxidation of NADPH is accompanied by an increased reduction of cytochrome P-450 were unsuccessful because the 40-80% fraction contained material which bound carbon monoxide and exhibited an intense absorption maximum at 420 μm interfering with the measurement of cytochrome P-450 (Table VIII). However, Kimura (60) has shown that adrenodoxin is required for reduction of cytochrome P-420.

The effect of menadione on NADPH oxidation was next examined.(Fig. 27a). The presence of menadione (72 μmoles/l, final concentration) increases the rate of oxidation of NADPH from 2.8 μmoles/min to 59 μmoles/min, a 21-fold increase. It is therefore evident that menadione can act as
Fig. 27a. Effect of menadione on the oxidation of NADPH by the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

The 20-40% ammonium sulfate precipitate obtained from 4 ml of enzyme preparation was dissolved in a solution made up of Tris-MgCl₂ buffer (0.6 ml) and 0.154 M KCl (1.0 ml). Propylene glycol (0.04 ml), and menadione (72 µmoles/l, final concentration) dissolved in 0.02 ml of ethanol were added and the mixture was divided equally between two cuvettes. After pre-incubation at 25° C for 6 minutes, NADPH (0.27 µmole) dissolved in 0.01 ml of Tris-MgCl₂ buffer was added to the sample cuvette. The control incubation received 0.01 ml of ethanol instead of menadione.

Control ( ); plus menadione ( ).

Fig. 27b. Effect of menadione on the NADPH mediated reduction of cytochromes P-450 and P-420 in the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

The reaction mixture in each cuvette was as described in Fig. 27a except that NADPH (0.27 µmole) dissolved in 0.01 ml of Tris-MgCl₂ buffer was added to both the sample and reference cuvettes. After 3 minutes of incubation, carbon monoxide was bubbled through the sample cuvette solution for 45 seconds and the carbon monoxide difference spectrum was recorded.

Control ( ); plus menadione ( ).
an electron acceptor in the 20-40% ammonium sulfate fraction, a finding in agreement with that of Kimura (60). The effect of this stimulation of NADPH oxidation by menadione on the reduction of cytochromes P-450 and P-420 is shown in Figure 27b. There is a slight decrease in the extent of cytochrome P-450 reduction on addition of menadione. However, menadione addition produces a substantial increase in cytochrome P-420 reduction as indicated by the greater than two-fold increase in the absorption maximum at 420 μm. Therefore, cytochrome P-420 is capable of acting as an electron acceptor in the presence of menadione. In the 20-40% ammonium sulfate fraction menadione is, in some manner, mediating the NADPH-linked reduction of cytochrome P-420 by adrenodoxin reductase, perhaps acting as an intermediate electron acceptor. Nishibayashi et al. have observed (147) that the NADPH-naphthoquinone reductase of rabbit liver microsomes could not utilize cytochrome P-420 as an electron acceptor. This is also true with the system from adrenal mitochondria. Very little oxidation of NADPH or reduction of cytochrome P-420 takes place when both the NADPH reductase and cytochrome P-420 are present in the 20-40% ammonium sulfate fraction (Table VIII, Fig. 27b). However the presence of menadione allows the transfer of electrons from NADPH to cytochrome P-420 in the system extracted from an adrenal mitochondrial acetone powder.

(d) Examination of the Acetone-Extracted Lipid Factors Affecting NADPH Oxidation and 11β-Hydroxylation

Studies with the acetone-extracted lipid fraction showed
that it affected both NADPH oxidation and 11β-hydroxylation (Figs. 21 and 26). The effect of the acetone-extracted lipid fraction on NADPH oxidation was very similar to the effect produced by menadione (Fig. 22). However, menadione produced no stimulation of 11β-hydroxylation. A preliminary fractionation of the acetone-extracted lipid was undertaken in an attempt to resolve and purify the factors or components responsible for the effects on NADPH oxidation and 11β-hydroxylation.

Water was removed from the acetone-extracted lipid fraction by azeotropic evaporation with absolute ethanol. The dried material was ground to a fine powder and extracted with petroleum ether. The petroleum ether fraction was concentrated under reduced pressure and the concentrate was then stirred into acetone and maintained at -20° C overnight. The phospholipid fraction was then obtained as a precipitate (111). All fractions were evaporated almost to dryness in vacuo, and re-suspended in Tris buffer by ultrasonic treatment. Portions of each fraction were then examined for their effect on both 11β-hydroxylation and NADPH oxidation. The results of these studies are shown in Table IX. In order to exclude the possibility that NADPH could become rate-limiting and thus influence any effect on 11β-hydroxylation of the fractions to be tested, initial rates of 11β-hydroxylation and NADPH oxidation were measured (Table IX). The factors stimulating both NADPH oxidation and 11β-hydroxylation are largely extracted from the dried acetone-extracted lipid fraction with petroleum ether, although some stimulatory activity remains in the petroleum
TABLE IX

Effect of adrenal mitochondria lipid fractions on the 11β-hydroxylation of deoxycorticosterone and on NADPH oxidation

<table>
<thead>
<tr>
<th>Fraction added</th>
<th>Corticosterone synthesized</th>
<th>NADPH oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmole/l/min</td>
<td>Percent control activity</td>
</tr>
<tr>
<td>Control (no additions)</td>
<td>0.423</td>
<td>100</td>
</tr>
<tr>
<td>Total mitochondrial lipid</td>
<td>0.574</td>
<td>136</td>
</tr>
<tr>
<td>Petroleum ether insoluble</td>
<td>0.456</td>
<td>108</td>
</tr>
<tr>
<td>-20°C Acetone insoluble</td>
<td>0.552</td>
<td>130</td>
</tr>
<tr>
<td>-20°C Acetone soluble</td>
<td>0.435</td>
<td>103</td>
</tr>
<tr>
<td>Asolectin</td>
<td>0.485</td>
<td>115</td>
</tr>
</tbody>
</table>

Each fraction was suspended in Tris-MgCl₂ buffer at a concentration of 10 mg lipid/ml.

1) 11β-hydroxylation of deoxycorticosterone. Each reaction flask contained: deoxycorticosterone-1,2-³H (specific activity 6.74 x 10⁷ cpm/μmole) dissolved in 0.05 ml of propylene glycol to give a final concentration of 12.8 μmoles/l; 0.55 ml of Tris-MgCl₂ buffer; 1.3 ml of enzyme preparation; and 0.2 ml of the appropriate lipid fraction. After pre-incubation for 8 minutes, NADPH (2.2 μmoles) was added dissolved in 0.05 ml of Tris-MgCl₂ buffer. A sample (1.0 ml) was removed from each reaction flask after 90 seconds of incubation and pipetted into ethyl acetate (2 ml) to terminate the reaction.

2) NADPH oxidation. Each reaction flask contained: 0.05 ml of propylene glycol; 0.9 ml of Tris-MgCl₂ buffer; 1.95 ml of enzyme preparation; and 0.2 ml of the appropriate lipid fraction. NADPH (1.0 μmole) was added dissolved in 0.1 ml of Tris-MgCl₂ buffer at zero time.
ether-insoluble material. The 11β-hydroxylase stimulating activity is precipitated by acetone at -20°C suggesting that the factor producing this stimulation may be phospholipid in nature. NADPH oxidation stimulating activity is present in both the -20°C acetone soluble and precipitable fractions.

These results (Table IX) suggest that the factor(s) stimulating 11β-hydroxylation can be partially resolved from the factor(s) stimulating NADPH oxidation. All 11β-hydroxylase-stimulating activity is contained in a fraction precipitated by acetone at -20°C, a treatment known to precipitate phospholipids but not quinones (111). Some stimulation of 11β-hydroxylation can be achieved with asolectin, a commercial soybean phospholipid preparation, supporting the suggestion that phospholipid is responsible for the stimulation of 11β-hydroxylase activity (Table IX). Asolectin produces only an 80% increase in NADPH oxidation however much less than that produced by the -20°C acetone precipitate (3160%).

NADPH oxidation is almost equally stimulated by both the -20°C acetone soluble and insoluble fractions. The stimulation of NADPH oxidation by the -20°C acetone precipitate suggests that either there is contamination of this fraction with material from the soluble fraction, i.e., incomplete separation of the stimulating factor by this fractionation procedure, or that more than one component is responsible for the stimulation of NADPH oxidation. In any
case the stimulation of NADPH oxidation does not appear to be associated with the stimulation of 11β-hydroxylation as judged by the inability of the -20°C acetone soluble fraction to stimulate hydroxylation and by the relatively low stimulation of NADPH oxidation by asolectin, which does stimulate 11β-hydroxylation.

Further examination of the lipids of beef adrenal mitochondria was undertaken in an attempt to isolate the factor stimulating NADPH oxidation by the enzyme preparation. The procedure used was a modification of that employed by Crane et al. (Ill) for the isolation of coenzyme Q₁₀ by direct solvent extraction of beef heart mitochondria. The mitochondria from 1000 g of beef adrenal glands were processed in this study. The ability of each isolated fraction to stimulate NADPH oxidation was measured and the results are shown in Table X. The fractions that stimulate NADPH oxidation are known to contain quinones (Ill). It is noted that the phospholipid fraction (-20°C acetone precipitate) does not stimulate NADPH oxidation, indicating a better separation of phospholipid from the factor stimulating NADPH oxidation than was achieved previously (Table IX). When the acetone-soluble fraction (Table X) was evaporated to dryness in vacuo and the material was redissolved in ethanol, white crystals formed on standing overnight at -20°C. These crystals were removed, dried, and their melting point was determined and found to be identical with an authentic sample of cholesterol. This fraction does not stimulate NADPH oxidation (Table X).
### TABLE X

Effect of adrenal mitochondria lipid fractions on NADPH oxidation

<table>
<thead>
<tr>
<th>Lipid Fraction added</th>
<th>Percent of control activity</th>
<th>Incubation time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Ethanol/ether soluble</td>
<td>457</td>
<td></td>
</tr>
<tr>
<td>Ethanol/ether insoluble</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Petroleum ether soluble</td>
<td>484</td>
<td></td>
</tr>
<tr>
<td>Petroleum ether insoluble</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>-20° C Acetone soluble</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>-20° C Acetone insoluble</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>-20° C Ethanol soluble</td>
<td>340</td>
<td></td>
</tr>
<tr>
<td>-20° C Ethanol insoluble</td>
<td>103</td>
<td></td>
</tr>
</tbody>
</table>

Each reaction flask contained: 1.05 ml of Tris-MgCl₂ buffer; 2.0 ml of enzyme preparation; 0.05 ml of lipid fraction suspended or dissolved in ethanol. NADPH (0.9 μmole) in 0.1 ml of Tris-MgCl₂ buffer was added at zero time.
The fractionation procedure outlined in Table X indicates that the factor that stimulates NADPH oxidation is extrac-
table from mitochondria with ethanol-ether, is soluble in
petroleum ether, acetone at -20° C, and ethanol at -20° C,
and is neither phospholipid nor cholesterol.

The material obtained from the -20° C ethanol-soluble
fraction was dissolved in a small amount of hexane and
applied to a silica gel column (1 cm x 19 cm). The column
was developed by a modification of the procedures described
by Crane et al. (111) and by Sottocasa and Crane (150). Each
eluate was examined for its ability to stimulate NADPH oxido-
dation. The results are shown in Table XI. Yellowish
material is eluted by each of the solvent combinations but
only those fractions eluted with 100% chloroform stimulate
the oxidation of NADPH. Crane et al. (111) have reported
that coenzyme Q₁₀ is eluted from silica gel with this sol-
vent. A portion of each column eluate was applied to a
silica gel thin-layer chromatography plate, and the plate
was developed with benzene. Only the three fractions that
stimulated NADPH oxidation had yellow, ultraviolet absorbing
zones, which exhibited an $R_f$ value corresponding to that
of an authentic sample of coenzyme Q₁₀. These zones appeared
as blue spots when sprayed with leucomethylene blue prepared
by the method of Crane and Dilley (112).

The absorption spectra of the eluates containing coenzyme
Q₁₀ exhibited a shoulder at 275 μm, the region of maximum ab-
sorption of quinones. However there was an increased
TABLE XI

Effect of silica gel column eluates on NADPH oxidation

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Eluent</th>
<th>Percent of control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Incubation time (minutes)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>Hexane</td>
<td>58</td>
</tr>
<tr>
<td>2</td>
<td>Hexane</td>
<td>114</td>
</tr>
<tr>
<td>3</td>
<td>Hexane</td>
<td>114</td>
</tr>
<tr>
<td>4</td>
<td>Hexane</td>
<td>73</td>
</tr>
<tr>
<td>5</td>
<td>Hexane</td>
<td>82</td>
</tr>
<tr>
<td>6</td>
<td>Hexane-chloroform (19:1)</td>
<td>82</td>
</tr>
<tr>
<td>7</td>
<td>Hexane-chloroform (9:1)</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>Hexane-chloroform (4:1)</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>Hexane-chloroform (1.5:1)</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>Chloroform</td>
<td>418</td>
</tr>
<tr>
<td>11</td>
<td>Chloroform</td>
<td>435</td>
</tr>
<tr>
<td>12</td>
<td>Chloroform</td>
<td>535</td>
</tr>
</tbody>
</table>

Incubations were carried out as described in Table X. 0.05 ml of each column eluate dissolved in ethanol was added to the reaction mixture.
absorption at shorter wavelengths indicating the presence of extraneous material. All attempts at further purification of the eluates stimulating NADPH oxidation resulted in a loss of this activity. Moreover, addition of authentic coenzyme Q\textsubscript{10} to the enzyme preparation did not produce any stimulation of NADPH oxidation. Because of the insolubility of coenzyme Q\textsubscript{10} in aqueous media it has been difficult to demonstrate that it functions in electron transport even though such a function has been implied (151-154). Experiments were therefore carried out to determine whether coenzyme Q\textsubscript{10} insolubility was responsible for its inability to stimulate NADPH oxidation in the 11\beta-hydroxylase preparation. Coenzyme Q\textsubscript{10} was added in the presence of Triton X-100, an agent used to solubilize quinones in electron transport studies, and in the form of a coenzyme Q-phospholipid micelle prepared with asolectin. Neither of these treatments resulted in stimulation of NADPH oxidation by coenzyme Q\textsubscript{10}. Therefore it is concluded that although coenzyme Q\textsubscript{10} is a component of the silica gel column eluates that stimulate NADPH oxidation, it alone may not be responsible for this stimulation. Other components of the neutral lipid fraction of mitochondria capable of reversible oxidation and reduction have been isolated (150). The possibility exists that the component responsible for this stimulation is merely eluted with coenzyme Q\textsubscript{10} and may be similar in chemical and physical properties to this quinone. Alternatively coenzyme Q may
act in conjunction with some other compound to stimulate NADPH oxidation.

Experiments were carried out to determine whether the oxidation of NADPH by the enzyme preparation extracted from the adrenal mitochondrial acetone powder was due to the presence of a bound quinone-type compound. Four g of mitochondrial acetone powder were saponified with 8 g NaOH, 2 g pyrogallol, 40 ml methanol and 30 ml of H₂O for one hour (113). The refluxed mixture was extracted with hexane, the hexane fraction was concentrated and applied to a magnesol-celite (5:1) column. The column was developed as described by Bragg and Polglase (114). The absorption spectra of the eluate fractions gave no indication of the presence of vitamin K or coenzyme Q. Therefore, within the limits of the method employed, it must be concluded that the oxidation of NADPH by the enzyme preparation is not mediated by a quinone.

(e) Stimulation of 11β-Hydroxylation by the Acetone-Extracted Lipid Fraction and by Asolectin

The fractionation studies outlined in Table IX suggest that a phospholipid present in this acetone extract is responsible for the stimulation of 11β-hydroxylation. A comparison of the effects of the acetone-extracted lipid and asolectin, a commercial plant phospholipid preparation, on 11β-hydroxylation was therefore carried out.
The effects of the acetone-extracted lipid fraction and asolectin on the course of 11β-hydroxylation are shown in Figure 28. Addition of the acetone-extracted lipid (2.4 mg/ml, final concentration) results in a 1.7-fold increase in the initial rate of 11β-hydroxylation. In addition, the rate of hydroxylation in the presence of lipid remains linear throughout the course of incubation, while in the control the rate decreases in the latter stages of incubation. This result suggests that the lipid fraction is stabilizing a labile component required for hydroxylation. The stimulation of 11β-hydroxylation by the acetone-extract can also be produced by asolectin. This phospholipid preparation, at a concentration equivalent to that of the acetone-extracted lipid (2.4 mg/ml), produces a 1.4-fold increase in the initial rate of 11β-hydroxylation.

The stimulation of 11β-hydroxylation as a function of lipid concentration is shown in Figure 29. Addition of increasing quantities of the acetone-extract lipid fraction results in an increase in 11β-hydroxylation, a maximal stimulation of 30% being achieved at a lipid concentration of 1 mg/ml. Asolectin, however, produces 55% stimulation at the lowest concentration tested (0.25 mg/ml) and further increases in asolectin concentration cause a diminution in its stimulatory effect. This loss of stimulatory activity at higher asolectin concentrations suggests that the phospholipid preparation contains a component that inhibits 11β-hydroxylase activity at higher concentrations of the phospholipid preparation.
Fig. 28. Effect of mitochondrial lipid and asolectin on the 11β-hydroxylation of deoxycorticosterone.

Each reaction flask contained: deoxycorticosterone-1,2-\(^{3}\)H (specific activity 5.63 \(\times\) 10\(^6\) cpm/\(\mu\)mole) dissolved in 0.05 ml of propylene glycol, 54 \(\mu\)moles/l, final concentration; 1.0 ml of Tris-MgCl\(_2\) buffer; 2.0 ml of enzyme preparation; and 0.2 ml of lipid suspension containing 40 mg lipid/ml. After pre-incubation for 8 minutes, NADPH (4.4 \(\mu\)moles) dissolved in 0.1 ml Tris-MgCl\(_2\) buffer was added. A sample (1.0 ml) was removed from each reaction flask after 8, 16, and 24 minutes of incubation and pipetted into ethyl acetate (2 ml) to terminate the reaction.

Control (○); plus acetone-extracted lipid (△); plus asolectin (○).
Incubations were carried out as described in Table IX. Deoxycorticosterone-1,2-\(^3\)H (specific activity 4.58 x 10\(^7\) cpm/\(\mu\)mole) dissolved in 0.05 ml of propylene glycol was added to each reaction flask to give a final concentration of 12.8 \(\mu\)moles/l. Mitochondrial lipid or asolectin (0, 0.5, 1.0, 2.0, 4.0 or 8.0 mg) was added suspended in 0.2 ml of Tris-MgCl\(_2\) buffer. Acetone-extracted lipid (○); asolectin (●).
Cytochrome P-450 possesses hydrophobic properties due to its phospholipid content or environment (90,93,94). Therefore the possibility that the phospholipid stimulation of 11β-hydroxylation was a result of some effect on cytochrome P-450 was deemed worthy of examination. The effect of the acetone-extracted lipid fraction on cytochrome P-450 in both the presence and absence of deoxycorticosterone substrate is shown in Figure 30. The cytochrome was measured under experimental conditions identical with those employed for measurement of 11β-hydroxylase activity shown in Figure 29. Addition of deoxycorticosterone (12.7 μmoles/l, final concentration) increases the recovery of cytochrome P-450 as has been previously demonstrated (Fig. 17). In the presence of the acetone-extracted lipid fraction an increase in cytochrome P-450 is observed which is greater than that produced by the substrate deoxycorticosterone. No additional increase in cytochrome P-450 is noted when deoxycorticosterone and lipid are both present in the reaction mixture. Thus the effects of deoxycorticosterone and the acetone-extracted lipid on cytochrome P-450 are not additive. The increase in cytochrome P-450 produced by the acetone-lipid fraction can explain the stimulation of 11β-hydroxylation by this fraction observed in Figures 28, 29 and Table IX.

The factor present in the acetone-extracted lipid fraction producing the stimulation in 11β-hydroxylase activity is precipitated from acetone at -20°C (Table IX). The data shown in Figure 31 reveal that increased cytochrome
Fig. 30. Effect of deoxycorticosterone and mitochondrial lipid on cytochrome P-450.

Appropriate reaction flasks contained: deoxycorticosterone (12.7 μmoles/l, final concentration) dissolved in 0.15 ml of propylene glycol; 1.65 ml of Tris-MgCl₂ buffer, 3.90 ml of enzyme preparation; and 0.6 ml of a lipid suspension (10 mg lipid/ml). After 8 minutes pre-incubation, NADPH (3 μmoles) was added dissolved in 0.15 ml of Tris-MgCl₂ buffer to each reaction flask. Solid ammonium sulfate to give 20% saturation was then added after 90 seconds of incubation and cytochrome P-450 was isolated and measured as described in the methods.

Without deoxycorticosterone, clear bars; plus deoxycorticosterone, shaded bars.
Incubations were carried out as described in Fig. 30. 0.15 ml of propylene glycol was added to each reaction flask instead of deoxycorticosterone. The lipid fractions (6 mg) were added suspended in Tris-MgCl$_2$ buffer (0.6 ml).

Cytochrome P-450, clear bars; cytochrome P-420, shaded bars.

Fig. 31. Effect of total mitochondrial lipid and -20°C acetone-insoluble fraction on cytochromes P-450 and P-420.
P-450 levels can also be produced by this precipitated factor, although to a slightly lesser degree than that produced with the total lipid fraction. This would indicate that the phospholipid present in the lipid fraction is responsible for the elevation in cytochrome P-450 content.

Figure 32 shows that the increase in cytochrome P-450 recovered after the incubation period is dependent upon the concentration of the acetone-extracted lipid fraction present in the reaction mixture. The effect of increasing concentrations of lipid on cytochrome P-450 (Fig. 32) is similar to the effect of lipid on 11β-hydroxylation (Fig. 29), both effects being maximal at a lipid concentration of approximately 1 mg/ml. The similarity of the two curves suggests that the observed stimulation of 11β-hydroxylation results from increased cytochrome P-450. Cytochrome P-420 is also increased in the presence of the lipid extract (Fig. 32) although to a lesser extent.

Asolectin produces increases in cytochrome P-450 levels at concentrations lower than the lipid extract, a two-fold increase in cytochrome P-450 being observed in the presence of asolectin at a concentration of 0.23 mg/ml (Fig. 33). As the asolectin concentration increases, cytochrome P-450 diminishes. A similar effect of asolectin is seen on cytochrome P-420. The decrease in cytochrome P-450 at higher asolectin concentrations may be due to lysolecithin in the phospholipid preparation. Lysolecithin has detergent action and has been shown to cause the decomposition
Fig. 32. Recovery of cytochromes P-450 and P-420 as a function of the mitochondrial lipid concentration.

Each reaction flask contained: 0.15 ml of propylene glycol; 1.05 ml of Tris-MgCl$_2$ buffer; 3.90 ml of enzyme preparation; and 0.0, 0.15, 0.30, 0.60, or 1.2 ml of the mitochondrial lipid suspension (10 mg lipid/ml). The volume of each reaction mixture was adjusted to 6.3 ml with Tris-MgCl$_2$ buffer. After 8 minutes pre-incubation, NADPH (3 μmoles) was added dissolved in 0.15 ml of Tris-MgCl$_2$ buffer to each reaction flask. Solid ammonium sulfate was then added to give 20% saturation after 90 seconds of incubation and cytochromes P-450 and P-420 were isolated and measured as described in the methods.

Cytochrome P-450 (○); cytochrome P-420 (●).
Fig. 33. Recovery of cytochromes P-450 and P-420 as a function of asolectin concentration.

Incubations were carried out as described in Fig. 32. 0.0, 0.08, 0.15, or 0.6 ml of the asolectin suspension (10 mg asolectin/ml) was added to the reaction mixture.

Cytochrome P-450 (○); cytochrome P-420 (●).
of cytochrome P-450 to P-420. The decrease in cytochrome P-
450 produced by higher concentrations of asolectin is very
similar to the decrease in 11β-hydroxylase stimulation observed
at increased asolectin concentrations (Fig. 29) and indicates
a relationship between these two phenomena.

In order to establish the mechanism whereby the acetone-
extracted lipid fraction was producing an increased recovery
of cytochrome P-450 (Fig. 32), the rate of breakdown of cyto-
chrome P-450 was measured in the presence and absence of the
acetone-extracted lipid fraction. The results are illustrated
in Figure 34. Incubation of the enzyme preparation at 37° C
results in a decrease in recoverable cytochrome P-450 and a
corresponding increase in cytochrome P-420, as has been pre-
viously observed (Fig. 16). In the presence of the acetone-
extracted lipid fraction (0.93 mg/ml) there is an increase in
cytochrome P-450 recovery when compared to the control. The
increase in cytochrome P-450 in the zero-time control (main-
tained at 4° C) in the presence of the acetone-extracted
lipid is an unexpected finding. Previous evidence has indi-
cated that cytochrome P-450 is relatively stable at lower
temperatures (Fig. 15) and very little decomposition of the
hemoprotein would be expected at 4° C. The difference in
cytochrome P-450 levels at zero time may indicate that the
acetone-extracted lipid fraction is enabling reconstitution
of cytochrome P-450 from cytochrome P-420. The reconversion
of microsomal cytochrome P-420 to cytochrome P-450 has been
reported by Ichikawa and Yamano (94). The reconversion of
Fig. 34. Effect of mitochondrial lipid on the rate of cytochrome P-450 disappearance.

Appropriate reaction flasks contained: 0.15 ml of propylene glycol; 1.65 ml of Tris-MgCl₂ buffer; 3.90 ml of enzyme preparation and 0.6 ml of a mitochondrial lipid suspension (10 mg lipid/ml). Solid ammonium sulfate was added to give 20% saturation after 0, 4, 8 and 12 minutes of incubation and cytochromes P-450 and P-420 were isolated and measured as described in the methods.

Cytochrome P-450: control (○--○); plus mitochondrial lipid (○—○). Cytochrome P-420: control (○--○); plus mitochondrial lipid (○—○).
detergent-produced cytochrome P-420 to cytochrome P-450 was produced by polyols such as glycerol or ethylene glycol, and at temperatures as low as 0°C. The possibility that the acetone-extracted lipid fraction is achieving a similar reconversion of cytochrome P-420 to cytochrome P-450 in the enzyme system from an adrenal mitochondrial acetone powder cannot be disregarded. Incubation of the enzyme preparation in the presence of acetone-extracted lipid, at 37°C, results in an initial rapid decrease in cytochrome P-450 to the level of the control incubation at zero time (Fig. 34). Thus the reconstituted cytochrome P-450 formed in the presence of lipid must be extremely labile and undergo spontaneous decomposition at elevated temperatures. Upon further incubation however, there is no additional loss of cytochrome P-450 in the presence of the acetone-extracted lipid (Fig. 34). Thus this lipid fraction must also stabilize cytochrome P-450.

DISCUSSION

The inhibition of 11β-hydroxylation by dicumarol suggested the involvement of a quinone-type compound in the hydroxylation reaction, since dicumarol is known to effect a number of pyridine nucleotide-quinone reductases (135-141). The enzyme preparation extracted from an acetone powder of adrenal mitochondria does indeed exhibit a naphthoquinone-stimulated oxidation of NADPH. This NADPH-quinone reductase activity has properties similar to those of microsomal NADPH-cytochrome c reductase (62).
Both menadione (2-methyl-1,4-naphthoquinone) and 1,4-naphthoquinone stimulate the aerobic oxidation of NADPH by the mitochondrial enzyme system, but 1,4-toluquinone and 1,4-benzoquinone are relatively ineffective. Similar specificity has been reported in other quinone-dependent NADPH oxidases (136,137,155). However, dicumarol stimulates the quinone-dependent activity in the adrenal mitochondrial acetone powder preparation. Dicumarol must be releasing or affecting some factor involved in this oxidation reaction which results in an extensive increase in NADPH oxidation in the presence of naphthoquinones. In contrast, the quinone reductase of the microsomal and soluble fractions of liver have been reported to be either inhibited by (136,137) or insensitive to (146) dicumarol.

Although naphthoquinones stimulate the oxidation of NADPH, they do not have this effect on 11β-hydroxylation. Indeed, under conditions where NADPH is rate-limiting, menadione can actually produce an inhibition of 11β-hydroxylation. Thus, there must be a competition between menadione and the 11β-hydroxylase for NADPH. A site for the interaction of menadione with the 11β-hydroxylase pathway was first suggested by Kimura (60) when he observed that adrenodoxin reductase, a constituent of the 11β-hydroxylase pathway, could utilize electron acceptors such as cytochrome c, menadione, and ferricyanide in place of the natural acceptor, adrenodoxin. Similar observations have been made in present studies. Moreover, cytochrome P-420 can in turn accept electrons from menadione,
or menadione can function as a cofactor in a NADPH-cytochrome P-420 reductase present in this enzyme system. A summary of the interaction of menadione with the 11β-hydroxylase pathway may be outlined as shown in Figure 35. Electrons can be transferred from NADPH via adrenodoxin reductase and menadione to cytochrome P-420, which can interact with oxygen to regenerate the oxidized form of the cytochrome (89). Menadione is also autoxidizable (146,147). Thus the possibility that the oxidized form of this quinone is regenerated by interaction with molecular oxygen as well as with cytochrome P-420 cannot be disregarded. The scheme presented here (Fig. 35) illustrates the possible catalytic role of menadione in NADPH oxidation, explaining its ability to rapidly oxidize quantities of NADPH (0.6 μmole) in excess of that expected for the reduction of the added menadione (0.05 μmole) (Fig. 22).

The lack of effect of menadione on 11β-hydroxylation in the presence of excess NADPH indicates its non-involvement in this reaction sequence and suggests that dicumarol inhibition of the 11β-hydroxylase is not mediated by its effect on this type of reaction. Indeed, addition of dicumarol to the incubation mixture in the presence of menadione results in an increased inhibition of 11β-hydroxylation due to the extremely rapid oxidation of NADPH in the presence of both dicumarol and menadione.

Kimura also observed (60) that adrenodoxin reductase may utilize cytochrome c as an electron acceptor instead of adrenodoxin. Omura et al. (57) reported that purified adrenodoxin
Fig. 35. Schematic representation of the interaction of menadione with the components of the electron transport pathway for 11β-hydroxylation.
reductase failed to catalyze the reduction of cytochrome c by NADPH unless adrenodoxin was also present. Spectrophotometric evidence indicates the presence of NADPH-cytochrome c reductase activity in the enzyme preparation employed in the present studies (Fig. 7). This reduction of cytochrome c is likely mediated by the adrenodoxin reductase described by Kimura (60) and Omura et al. (57).

The stimulation of NADPH oxidation by the acetone-extracted lipid fraction is very similar to that observed with naphthoquinones (Figs. 22 and 23). Both stimulatory processes are affected by dicumarol in an analogous manner (Table VII). However, whether the stimulation of NADPH oxidation by the acetone-extracted lipid fraction is due to the presence of a quinone cannot be stated with certainty. Attempts to isolate the stimulatory factor from the lipid extract were inconclusive. All activity was found to reside in fractions containing coenzyme Q_{10} but further attempts at purification resulted in a loss of stimulatory activity. Furthermore, addition of authentic coenzyme Q_{10} to the enzyme preparation under a variety of conditions employed to solubilize the quinone did not result in any significant stimulation of NADPH oxidation. Sottocasa and Crane (150) have isolated four components of the neutral lipid fraction of beef heart mitochondria capable of undergoing reversible oxidation and reduction. One of these components was found to be coenzyme Q_{10}, another was found in the fraction containing \alpha-tocopherol, while the nature of the other two components could
not be clearly established. Sottocasa and Crane observed that one unknown component behaved chromatographically similar to known members of the vitamin K family, while the other had spectral properties suggesting a benzoquinone type of structure. Of interest is the statement of Sottocasa and Crane that this component exhibited a spectrum similar to that of coenzyme \(Q_{10}\) and was not easily separated from coenzyme \(Q_{10}\) on silicic acid column chromatography. It is possible that a similar component is present in the lipid extract of adrenal mitochondria and that it is responsible for the observed stimulation of NADPH oxidation.

The stimulation of \(11\beta\)-hydroxylation by the acetone-extracted lipid fraction may be attributed to the phospholipid components of the lipid extract. The stimulatory factor can be precipitated from acetone at \(-20^\circ\) C and its activity can be partially mimicked by asolectin, a soybean phospholipid preparation. The stimulatory effect of this phospholipid component of the acetone-extracted lipid may be due to stabilization of cytochrome P-450. Moreover, the phospholipid may produce a reconstitution of cytochrome P-450 from cytochrome P-420 although the reconstituted hemoprotein appears to be extremely labile. Thus the acetone-extracted lipid fraction contains the lipid moiety of cytochrome P-450 removed during preparation of the acetone powder; and recombination of this moiety with cytochrome P-420 produces the cytochrome P-450 complex. Polyols such as glycerol can protect cytochrome P-450 from decomposition (80,94) and effect
a reconstitution of this hemoprotein from cytochrome P-420 (94). The effects of polyols are analogous to the phospho-
lipid effect on cytochrome P-450 demonstrated in the present
experiments.

The mechanism whereby phospholipid stabilizes cytochrome
P-450 is unclear. This hemoprotein contains phospholipid and
requires phospholipid to maintain its unique properties. The
importance of lipid in the function of the mitochondrion has
been the subject of much research (110,152,156). Green and
Tzagoloff (156) have postulated that phospholipid micelles
can interact with basic proteins such as cytochrome c to form
stable complexes. Cytochrome P-450 may well be such a com-
plex and the presence of additional phospholipid may aid in
stabilizing the cytochrome P-450 complex as well as recon-
stituting cytochrome P-450 from cytochrome P-420 produced by
removal of phospholipid by acetone. The requirement of
certain enzymes located in the inner membrane of the mito-
chondrion for phospholipid has been established (156).
Examples are the phospholipid requirement of the electron
transport chain (152), \( \beta \)-hydroxybutyrate dehydrogenase (157,
158), and cytidine diphosphocholine tranferase (156,159).
The particulate nature of mitochondrial cytochrome P-450 and
the interaction of the 11\( \beta \)-hydroxylase pathway with the
classical electron transport chain suggest that this hemo-
protein may also be located in the inner membrane of the
mitochondrion and thus may exhibit a similar phospholipid
requirement.
The inability to demonstrate that a quinone-dependent NADPH reductase functions in 11β-hydroxylation suggests that dicumarol inhibition of 11β-hydroxylation does not proceed via its effect on a quinone reductase reaction functioning in 11β-hydroxylation. While a phospholipid component of the lipid extract of adrenal mitochondria can stimulate 11β-hydroxylation, this component does not significantly overcome the inhibition produced by dicumarol under these experimental conditions (Fig. 26). Therefore dicumarol inhibition of 11β-hydroxylation must be achieved by a mechanism not yet ascribed to this compound.
Evidence presented in Part II of this thesis excluded the possibility that the inhibition of $11\beta$-hydroxylation by dicumarol was produced by an inhibitory action of this compound on a NADPH-quinone reductase. Hence a detailed kinetic examination of dicumarol inhibition was undertaken in an attempt to determine its mode of action. Similar studies on the mechanism of inhibition of Metopirone, a well established inhibitor of $11\beta$-hydroxylation (42-49), were also carried out. It was hoped that a comparative study of the two inhibitors would lead to an understanding of their modes of action and of the general mechanism of $11\beta$-hydroxylation.

RESULTS

(a) Inhibition of $11\beta$-hydroxylation by Dicumarol

Figure 36a shows the rates of oxidation of NADPH by the enzyme preparation alone, and in the presence of deoxycorticosterone, dicumarol, and deoxycorticosterone plus dicumarol. Addition of deoxycorticosterone to the reaction mixture increases the rate of NADPH oxidation over that of the control. The addition of dicumarol to the enzyme preparation also causes an increase in the rate of NADPH oxidation. However, no further enhancement of NADPH oxidation is observed in the presence of dicumarol plus deoxycorticosterone. These findings indicate that dicumarol inhibits the oxidation of that portion of the reduced coenzyme participating in steroid $11\beta$-
Fig. 36a. Effect of dicumarol and deoxycorticosterone on NADPH oxidation.

Appropriate reaction flasks contained: 0.6 μmole of deoxycorticosterone dissolved in 0.05 ml of propylene glycol; 1.0 ml of Tris-MgCl$_2$ buffer; 2.0 ml of enzyme preparation; and 0.5 μmole of dicumarol dissolved in 0.05 ml of a dilute alcoholic KOH solution. NADPH (0.8 μmole) in 0.1 ml of Tris-MgCl$_2$ buffer was added at zero time.

Control (o); plus deoxycorticosterone (o); plus dicumarol (o); plus deoxycorticosterone and dicumarol (a).

Fig. 36b. Effect of dicumarol on the 11β-hydroxylation of deoxycorticosterone.

Incubations were carried out as described in Fig. 36a. 0.65 μmole of deoxycorticosterone-1,2-$^3$H (specific activity 1.07$x10^6$ cpmp/μmole) dissolved in 0.05 ml of propylene glycol was added to each reaction flask. After 8 minutes pre-incubation, NADPH (2.2 μmoles) dissolved in 0.1 ml of Tris-MgCl$_2$ buffer was added. A sample (1.0 ml) was removed from each flask after 8, 16, and 24 minutes incubation, and pipetted into ethyl acetate (2 ml) to terminate the reaction.

Control (o); plus dicumarol (a).
hydroxylation. The inhibitory action is confirmed by the data shown in Figure 36b. The formation of corticosterone is greatly reduced by the addition of dicumarol \(1.5 \times 10^{-4} \text{ M}\), being 37% that of the control after 8 minutes of incubation.

The concentration range over which dicumarol exhibits an inhibitory action on \(11\beta\)-hydroxylation is shown in Figure 37. Inhibition by this compound is demonstrable at concentrations of dicumarol as low as 11.6 \(\mu\)moles/l. At the lower dicumarol concentrations \(11\beta\)-hydroxylation is relatively insensitive to the inhibitor, a slow linear increase in inhibition resulting from a thousandfold increase in dicumarol concentration. At the higher dicumarol concentrations there is a pronounced increase in the sensitivity of \(11\beta\)-hydroxylation to the inhibitor concentration. The data suggest that dicumarol exerts more than one mode of inhibition of the \(11\beta\)-hydroxylase system.

Kinetic experiments on the effect of dicumarol on the initial velocity of \(11\beta\)-hydroxylation were carried out. The effect of different dicumarol concentrations \((58, 145\) and \(233 \mu\)moles/l) on the initial rate of \(11\beta\)-hydroxylation at increasing substrate concentrations is kinetically represented in the plot of \([S]/v\) vs \([S]\) in Figure 38. At the lowest dicumarol concentration examined \((58 \mu\)moles/l) dicumarol is a noncompetitive inhibitor of \(11\beta\)-hydroxylation. However, increases in inhibitor concentration alter the inhibition by dicumarol to that of a mixed type, again suggesting more than one effect of dicumarol on \(11\beta\)-hydroxylation,
Fig. 37. Inhibition of the 11β-hydroxylation of deoxycorticosterone as a function of dicumarol concentration.

Each reaction flask contained: 0.094 μmole of deoxycorticosterone-1,2-3H (specific activity 1.05 × 10⁷ cpm/μmole) dissolved in 0.05 ml of propylene glycol; 1.35 ml of Tris-MgCl₂ buffer; 2.6 ml of enzyme preparation; dicumarol, to give the indicated final concentration, dissolved in 0.1 ml of dilute alcoholic KOH solution. After pre-incubation for 8 minutes, NADPH (3.3 μmoles) dissolved in 0.2 ml of Tris-MgCl₂ buffer was added. After 20 minutes of incubation, reactions were terminated by the addition of ethyl acetate (5 ml) to each flask.
Fig. 38. [S]/v versus [S] plot of dicumarol inhibition of the 11β-hydroxylation of deoxycorticosterone.

Each reaction flask contained: deoxycorticosterone-1,2-\(^3\)H (specific activity, 6.24 x 10\(^7\) cpm/μmole) dissolved in 0.05 ml of propylene glycol; 0.7 ml of Tris-MgCl\(_2\) buffer; 1.3 ml of enzyme preparation; 0, 0.125, 0.313, or 0.5 μmole of dicumarol dissolved in 0.05 ml of a dilute alcoholic KOH solution. After 8 minutes of pre-incubation, NADPH (1.7 μmoles) dissolved in 0.05 ml of Tris-MgCl\(_2\) buffer was added. A sample (1.0 ml) was removed from each reaction flask after 90 seconds of incubation and pipetted into ethyl acetate (2 ml) to terminate the reaction.

Final concentration (μmoles/l) of dicumarol: 0 (•), 58 (○), 145 (□), 233 (△).
the secondary effect being observed only at higher dicumarol concentrations. Also worthy of note is the fact that at high dicumarol concentrations, the experimental values determined cannot be as uniformly joined by a straight line as at the lowest dicumarol concentration or in its absence. It is possible that at high dicumarol concentrations the inhibitory effect is complex in nature and may involve several different interactions of dicumarol with the 11β-hydroxylase system.

That more than one inhibitory action of dicumarol on 11β-hydroxylation is possible is also demonstrated in the Dixon plot (160) of $1/v$ vs $i$ shown in Figure 39. The effect of increasing inhibitor concentrations at two levels of substrate (7 and 35 μmoles/l) is illustrated. At dicumarol concentrations of 80 μmoles/l and lower, the 11β-hydroxylase activity is relatively insensitive to changes in inhibitor concentration as judged by the gradients of the lines joining the experimental values. Extension of the lines obtained in this region of dicumarol concentration to their point of intersection can establish both the mode of inhibition and the $K_i$ for the inhibitor. However the almost horizontal slopes of the lines obtained at both substrate concentrations prevent an accurate determination of these parameters. Although one cannot conclude definitely from the Dixon plot (Fig. 39) that dicumarol at low concentrations is a noncompetitive inhibitor of 11β-hydroxylation, the kinetic data in Figure 38 indicate noncompetitive inhibition.
Fig. 39. $1/v$ versus $i$ plot of dicumarol inhibition of 11$\beta$-hydroxylation of deoxycorticosterone.

Incubations were carried out as described in Fig. 38. The specific activity of the deoxycorticosterone-1,2-$^3$H substrate was $4.04 \times 10^4$ cpm/µmole.

Final concentration (µmoles/l) of deoxycorticosterone-1,2-$^3$H: 7 (△), 35 (△).
The secondary effect of dicumarol on 11β-hydroxylation is evident in the Dixon plot (Fig. 39) where a distinct increase in the slope of the curve is apparent as the dicumarol concentration increases. At the higher deoxycorticosterone concentration, the point at which this slope change appears is at a slightly higher dicumarol concentration and the extent of the slope change is decreased. Thus the second dicumarol inhibitory effect is extremely sensitive to substrate concentration, being greatly diminished at a substrate concentration of 35 μmoles/l. This suggests that the second mode of dicumarol inhibition may be competitive with substrate.

(b) Inhibition of 11β-Hydroxylation by Metopirone

Since the introduction of Metopirone as an inhibitor of steroid biogenesis (42), various investigations have been conducted on the nature of this inhibition with regard to both specificity and mechanism of action. Inhibition of 11β-hydroxylation by Metopirone has been demonstrated both in vivo (44) and in vitro (45-49). Studies in our laboratory on the effects of Metopirone on 11β-hydroxylation and on the concomitant oxidation of NADPH were undertaken, and these effects were compared with those of dicumarol.

The effect of Metopirone on NADPH oxidation in the absence of steroid substrate is shown in Figure 40. The rate of oxidation of the coenzyme is enhanced by Metopirone, the increase being proportional to the quantity of Metopirone added. A three-fold increase in rate is observed in the
Fig. 40. NADPH oxidation as a function of Metopirone concentration.

Each reaction flask contained: 0.05 ml of propylene glycol; 1.0 ml of Tris-MgCl$_2$ buffer; 2.0 ml of enzyme preparation; and 0, 0.18, 0.36, or 0.72 μmole of Metopirone dissolved in 0.1 ml of Tris-MgCl$_2$ buffer. NADPH (0.8 μmole) dissolved in 0.05 ml of Tris MgCl$_2$ buffer was added at zero time.

Final concentration (μmoles/l) of Metopirone: 0 (○), 55.5 (○), 111 (▲), 222 (△).
presence of $2.2 \times 10^{-4}$ M Metopirone. The stimulation of NADPH oxidation by Metopirone is similar to that produced by dicumarol (Fig. 36a) in the absence of deoxycorticosterone, although Metopirone exhibits a greater stimulatory effect. Dicumarol ($1.56 \times 10^{-4}$ M) produces a 1.6-fold increase in NADPH oxidation, while Metopirone ($1.1 \times 10^{-4}$ M) produces a two-fold increase.

Preliminary examination of the inhibition of 11β-hydroxylation by Metopirone indicates that this compound effectively inhibits 11β-hydroxylation at concentrations much lower than those required to show a stimulation of NADPH oxidation (Fig. 41). In the presence of deoxycorticosterone (200 μmoles/l), 50% inhibition is attained at a Metopirone concentration of 4.2 μmoles/l, a value 26 times lower than that required to produce a two-fold stimulation of NADPH oxidation. The nature of the inhibition curve obtained with Metopirone differs from that obtained with dicumarol (Fig. 37). Metopirone effectively inhibits 11β-hydroxylation at lower concentrations and over a narrower concentration range of the inhibitor when compared to inhibition of 11β-hydroxylation by dicumarol.

The effect of Metopirone on NADPH oxidation in the absence and presence of substrate deoxycorticosterone is shown in Figure 42a. Low concentrations of Metopirone produce a slight increase in the rate of NADPH oxidation over that of the control. However, upon addition of deoxycorticosterone, the increase in NADPH oxidation in the presence of Metopirone is not as great as in its absence, suggesting an inhibitory
Fig. 41. Inhibition of the 11β-hydroxylation of deoxycortico­
costerone as a function of Metopirone concentration.

Incubations were carried out as described in Fig. 40. Deoxycorticosterone-1,2-³H, 0.67 μmole, (specific activity 2.19 x 10⁵ cpm/μmole) was added dissolved in 0.05 ml of propy­
lene glycol. After 5 minutes pre-incubation, NADPH (1.1 μmoles) was added dissolved in 0.05 ml of Tris-MgCl₂ buffer. After 20 minutes of incubation, reactions were terminated by the addition of chloroform (5 ml) to each flask.
effect of Metopirone on the hydroxylation reaction. This inhibition is confirmed by the data in Figure 42b showing the rate of formation of corticosterone from deoxycorticosterone. In the presence of Metopirone (2.7 μmoles/l, final concentration) there is a 46% decrease in the initial rate of 11β-hydroxylation. The slight stimulation of NADPH oxidation at Metopirone concentrations that effectively inhibit 11β-hydroxylation (Figs. 42a and 42b) indicates that this inhibition is not produced by a rapid utilization of NADPH present in the reaction mixture and that some other mechanism of inhibition must be involved.

An examination of the kinetics of Metopirone inhibition was carried out and the results are shown in Figure 43. In the Dixon plot of 1/v vs i, the lines joining the experimental values obtained at each substrate concentration intersect at a point above the abcissa corresponding to 1/V_{max} determined in the absence of inhibitor. The intersection of the lines at this point establishes that Metopirone inhibition is competitive in nature. The point of intersection also gives the value K_i of 1.0 x 10^{-7} moles/l. The competitive nature of Metopirone inhibition has also been demonstrated by Dominguez and Samuels (45) employing a whole homogenate of rat adrenal tissue. Thus Metopirone is a very potent inhibitor of 11β-hydroxylation, effectively inhibiting the reaction at concentrations much lower than the K_m of the substrate deoxycorticosterone (5.5 x 10^{-6} moles/l). The high affinity of Metopirone for the 11β-hydroxylase may explain the finding of
Fig. 42a. Effect of Metopirone and deoxycorticosterone on NADPH oxidation.

Incubations were carried out as described in Fig. 40. Deoxycorticosterone, 0.67 μmole, was added dissolved in 0.05 ml of propylene glycol. Metopirone, 0.013 μmole, was added dissolved in 0.1 ml of Tris-MgCl₂ buffer. NADPH (0.8 μmole) was added dissolved in 0.05 ml of Tris-MgCl₂ buffer at zero time. Control (o); plus deoxycorticosterone (●); plus Metopirone (●); plus Metopirone and deoxycorticosterone (□).

Fig. 42b. Effect of Metopirone on the 11β-hydroxylation of deoxycorticosterone.

Incubations were carried out as described in Fig. 40. Deoxycorticosterone-4-¹⁴C, 0.47 μmole, (specific activity, $6.63 \times 10^5$ cpm/μmole) was added dissolved in 0.05 ml of propylene glycol. Metopirone, 0.0088 μmole, was added dissolved in 0.1 ml of Tris-MgCl₂ buffer. After 5 minutes pre-incubation, NADPH (1.7 μmoles) was added dissolved in 0.05 ml of Tris-MgCl₂ buffer. A sample (1.0 ml) was removed from each reaction flask after 4, 8, and 12 minutes of incubation and pipetted into chloroform (2 ml) to terminate the reaction. Control (o), plus Metopirone (●).
Fig. 43. $1/v$ versus $i$ plot of Metopirone inhibition of the 11$\beta$-hydroxylation of deoxycorticosterone.

Each reaction flask contained: deoxycorticosterone-1, 2-$^3$H, (specific activity $7.2 \times 10^7$ cpm/µmole) dissolved in 0.05 ml of propylene glycol; 0.70 ml of Tris-MgCl$_2$ buffer; 1.30 ml of enzyme preparation; and Metopirone dissolved in 0.05 ml of Tris-MgCl$_2$ buffer. After 8 minutes pre-incubation, NADPH (1.7 µmoles) dissolved in 0.05 ml of Tris-MgCl$_2$ buffer was added. A sample (1.0 ml) was removed from each reaction flask after 90 seconds of incubation and pipetted into ethyl acetate (2 ml) to terminate the reaction.

Final concentration (µmoles/l) of deoxycorticosterone-1,2-$^3$H 3.35 (□), 10.2 (●), 30.5 (○).
Sharma et al (41) that Metopirone inhibition of steroid 11β-hydroxylation could not be relieved by increasing the substrate concentration. Under their experimental conditions, the greatest molar ratio of deoxycorticosterone:Metopirone examined was 4:1, a ratio too low to permit demonstrable reversal of inhibition.

Of particular interest is the kinetics obtained at the lowest deoxycorticosterone concentration, 3.35 μmoles/l (Fig. 43). At this substrate concentration, there is a change in slope of the curve as the Metopirone concentration is increased. This slope change is not observed at higher substrate concentrations. This change in slope is very similar to that obtained with dicumarol (Fig. 39). However the extent of the slope change in the presence of Metopirone is not as prominent as that obtained with dicumarol. The slope change in the presence of Metopirone is completely eliminated at a deoxycorticosterone concentration of 10 μmoles/l, while in the presence of dicumarol a small change is still observed at a deoxycorticosterone concentration of 35 μmoles/l. Furthermore, at low inhibitor concentrations Metopirone is a competitive inhibitor of 11β-hydroxylation, while dicumarol is a noncompetitive inhibitor. Thus it is unlikely that these inhibitors are acting by a similar mechanism. In both cases however more than one inhibitory effect can be noted, and this second effect appears to be very sensitive to the substrate concentration.
DISCUSSION

It is evident from these studies that the modes of action of dicumarol and Metopirone on \(11\beta\)-hydroxylation are different. Dicumarol appears to have two distinct inhibitory actions on \(11\beta\)-hydroxylation. Dicumarol at low concentrations, is a noncompetitive inhibitor. The inhibition produced at higher dicumarol concentrations is extremely sensitive to the substrate concentration. Metopirone, on the other hand, is a competitive inhibitor of \(11\beta\)-hydroxylation. There are indications that Metopirone may have more than one inhibitory action, but this is apparent only at very low substrate concentrations.

While the kinetics of inhibition by dicumarol and Metopirone are different, their effects on NADPH oxidation are similar. Both dicumarol and Metopirone produce an increase in the rate of NADPH oxidation in the absence of steroid substrate. This effect is more pronounced with Metopirone, a 3.2-fold increase in NADPH oxidation being produced at a Metopirone concentration of 222 \(\mu\)moles/l. Dicumarol, at a concentration of 157 \(\mu\)moles/l, produces a 1.5-fold increase in NADPH oxidation. Both inhibitors decrease the NADPH oxidation associated with steroid substrate hydroxylation.

Whether the stimulatory effect of these compounds on NADPH oxidation is associated with their inhibitory action on \(11\beta\)-hydroxylation is not clear. It is unlikely that \(11\beta\)-hydroxylation inhibition results from rapid and extensive
oxidation of NADPH by dicumarol or Metopirone. While Metopirone can produce a substantial increase in the rate of NADPH oxidation, this only occurs at concentrations much higher than that required to inhibit 11β-hydroxylation. Both dicumarol and Metopirone have oxo groups capable of undergoing reduction (Fig. 3). It is possible that the increased rate of NADPH oxidation observed in the presence of these compounds is due to the action of a dehydrogenase present in the enzyme preparation. Birmingham and Kraulis have observed that the rat adrenal gland reduces the oxo group of Metopirone to a hydroxyl function and that the reduced derivatives does not inhibit 11β-hydroxylation (161). Metabolites of dicumarol have also been isolated from the urine of dicumarol-treated rats (162). Alternatively these inhibitors could be acting as electron acceptors to an enzyme in the 11β-hydroxylase pathway thus interrupting the flow of electrons to the site of deoxycorticosterone hydroxylation.

Kinetic examination of dicumarol inhibition of 11β-hydroxylation reveals that this compound has more than one mode of action. At low dicumarol concentrations, inhibition of 11β-hydroxylation is relatively insensitive to variations in dicumarol concentration and is noncompetitive with substrate deoxycorticosterone. At high dicumarol concentrations, inhibition of 11β-hydroxylation is extremely sensitive to variations in dicumarol concentration. In addition, this secondary effect of dicumarol can be greatly diminished by increasing the substrate deoxycorticosterone concentration
suggesting a possible competition of substrate and inhibitor for the 11β-hydroxylase at the higher dicumarol concentrations.

Although nonlinear kinetics are often associated with allosteric enzymes (163-167), deviations from Michaelis-Menton kinetics need not necessarily imply allosteric processes. Gawron et al. (168) suggest that nonlinear kinetics can be explained in terms of a multistage reaction sequence. Datta and Prakash (169) in studies with the aspartokinase of Rhodospseudomonas spheroides observed that reciprocal plots of \( \frac{1}{v} \) vs \( \frac{1}{[ATP]} \) yielded biphasic curves that they attributed to two binding sites on the enzyme molecule for ATP. The biphasic curves obtained in the \( \frac{1}{v} \) vs \( i \) plot (Fig. 39) demonstrating dicumarol inhibition of 11β-hydroxylation may likewise indicate two distinct inhibitory sites on a member enzyme of the 11β-hydroxylase pathway for this compound.

Several explanations of the complex inhibition of 11β-hydroxylation by dicumarol may be proposed. 11β-hydroxylation requires the interaction of steroid substrate, molecular oxygen, and the reducing equivalents of NADPH for the introduction of a hydroxyl function into the steroid nucleus. At low concentrations, dicumarol may alter the interaction of these factors, perhaps by inducing a conformational change in the enzyme catalyzing this interaction. Such an alteration could lead to a reduced rate of 11β-hydroxylation without affecting the binding of the steroid substrates to the enzyme molecule. Such a mechanism of dicumarol inhibition would be noncompetitive with regard to steroid substrate, and this is
indeed found to be the case at low dicumarol concentrations (Fig. 38). Alternatively dicumarol at low concentrations could be affecting the binding of molecular oxygen to cytochrome P-450. Again, increasing the steroid substrate concentration would not overcome this inhibition. Datta (170) has pointed out that in reactions involving more than one substrate, modifiers may influence the binding of one substrate and not influence that of the others. Evidence now suggests that 11β-hydroxylation involves the sequential interaction of at least four components in the form of an electron transport chain. These components are NADPH, adrenodoxin reductase, adrenodoxin, and cytochrome P-450. Interaction of dicumarol with a component of this chain prior to that actually involved in steroid binding would result in the noncompetitive type of inhibition that is observed.

The secondary inhibitory effect of dicumarol at higher concentrations is sensitive to steroid substrate (Fig. 39). This suggests that at these concentrations, dicumarol is interfering with the actual binding of deoxycorticosterone to the 11β-hydroxylase component, cytochrome P-450. Whether this secondary inhibition is competitive or noncompetitive in nature cannot be unequivocally established because this effect is superimposed on the noncompetitive inhibition produced at the lower dicumarol concentrations. The observed inhibition by higher dicumarol concentrations is of a mixed type (Fig. 38). Dicumarol could be interfering with the
binding of deoxycorticosterone to cytochrome P-450 by itself binding to the substrate site, or by inducing a conformational alteration in the enzyme that precludes binding of the steroid substrate.

Although the two observed effects of dicumarol on 11β-hydroxylation may be separate and distinct, the possibility exists that they are interrelated. A conformational alteration produced by the binding of the first dicumarol molecule may be required before the second dicumarol molecule can bind to the second site. The mechanism of dicumarol inhibition cannot be clearly established without a more detailed study of the interaction of dicumarol with the components of the 11β-hydroxylase pathway.

In contrast to dicumarol, Metopirone is a competitive inhibitor of 11β-hydroxylation (Fig. 43). Metopirone inhibits 11β-hydroxylation at concentrations lower than the \( K_m \) of substrate deoxycorticosterone, while at comparable dicumarol concentrations, the inhibition of 11β-hydroxylation is less extensive (Figs. 37 and 41).

The structural features of Metopirone that permit competitive inhibition of deoxycorticosterone 11β-hydroxylation are unclear. Dominguez and Samuels (45) suggest that there is a structural relationship between the corticosteroid side-chain and Metopirone, and that the inhibitor may be interfering with the interaction of the \( \alpha \)-ketol sidechain with the enzyme to which it binds. However, Sharma et al. (41) have reported that androstenedione, a steroid without an \( \alpha \)-ketol
sidechain, is a competitive inhibitor of 11β-hydroxylation of deoxycorticosterone. Thus the importance of the sidechain in the binding of deoxycorticosterone to the enzyme or in the specificity of the 11β-hydroxylase is questionable. The elucidation of the structural features of Metopirone that permit inhibition of 11β-hydroxylation must await assignment of the requisite structural aspects of the steroid molecule involved in enzyme binding and specificity of the 11β-hydroxylase.

The nature of competitive inhibition is usually explained in terms of competition of the inhibitor and substrate for the same site on the enzyme (i.e., isosteric inhibition). However it is known (171) that although an inhibition may follow precise competitive kinetics, this does not necessarily imply that it competes with the substrate for the same binding site. The inhibitor-binding site on the enzyme may be near or may partially overlap the substrate binding site. Hence binding of the inhibitor may alter the conformation of the active site and thus affect the affinity of the substrate for the enzyme (172). Metopirone may function as a competitive inhibitor of the 11β-hydroxylase in this manner.

The data presented in Figure 43 show that at low substrate concentrations (3.35 μmoles/l) the kinetic curve of Metopirone inhibition is biphasic. This may indicate that Metopirone is capable of inducing a conformational alteration in the enzyme to which it binds that results in an increase in the effectiveness of Metopirone as an inhibitor. As this secondary effect is not apparent at higher substrate concentration, it is
concluded that deoxycorticosterone can prevent this conformational alteration with the result that normal Michaelis-Menten kinetic are observed. A more detailed study of Metopirone inhibition at low substrate concentrations is required before the significance of the biphasic nature of Metopirone inhibition at low substrate concentrations can be evaluated.
PART IV  EFFECT OF DICUMAROL AND METOPIRONE ON CYTOCHROMES P-450 AND P-420

Evidence suggests that cytochrome P-450 is the oxygen-activating component of mixed function oxidase systems (67, 68, 72, 76). This hemoprotein may be responsible also for substrate binding and activation (95-98). The possibility that dicumarol and Metopirone inhibition of 11β-hydroxylation is a result of an interaction of these compounds with cytochrome P-450 was therefore deemed worthy of study. The unique absorption spectrum produced on binding of carbon monoxide to cytochrome P-450 permits examination of this hemoprotein without prior extensive purification. The effects of dicumarol and Metopirone on cytochrome P-450 were investigated under experimental conditions similar to those of the kinetic experiments.

RESULTS

(a) Effect of Dicumarol on Cytochromes P-450 and P-420

The enzyme preparation was incubated in the presence of dicumarol at two inhibitor concentrations, but in the absence of deoxycorticosterone substrate. One dicumarol concentration (23 µmoles/l) was in the region of noncompetitive inhibition of 11β-hydroxylation (Figs. 38 and 39) while the second (116 µmoles/l) was in the region producing inhibition of a mixed nature. Incubation conditions were the same as those employed in the kinetic studies. The reaction mixture was pre-incubated
at 37° C for 8 minutes in the presence of dicumarol. NADPH was then added, and the reaction continued for an additional 90 seconds. The reaction mixture was then fractionated (at 4° C) with ammonium sulfate and the carbon monoxide difference spectrum of the fraction precipitating between 20% and 40% saturation with ammonium sulfate was determined. These spectra are illustrated in Figure 44. The spectrum of the control incubation (no dicumarol) shows the presence of both cytochromes P-450 and P-420. In the presence of the lower dicumarol concentration (23 μmoles/l) the amount of cytochrome P-450 present in this ammonium sulfate fraction is twice that of the control incubation. There is a slight increase in cytochrome P-420 as well. However, addition of dicumarol at the higher concentration (116 μmoles/l) yields almost entirely cytochrome P-420. These results suggest that dicumarol may have more than one effect on cytochrome P-450. A more extensive study on the effect of dicumarol concentration on cytochromes P-450 and P-420 was carried out under the same conditions as outlined in Figure 44. The effect of very low dicumarol concentrations on cytochromes P-450 and P-420 is shown in Figure 45. At dicumarol concentrations as low as 2.3 μmoles/l there is a significant increase in the cytochrome P-450 levels. In addition, at very low dicumarol concentrations, 0.23 to 2.3 μmoles/l, there is also an increase in cytochrome P-420. As the dicumarol concentration is increased to 11.6 μmoles/l, the cytochrome P-420 content diminishes. Although the presence of dicumarol at low concentrations
Fig. 44. Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of dicumarol.

Appropriate reaction flasks contained: 0.15 ml of propylene glycol; 2.1 ml of Tris-MgCl$_2$ buffer; 3.9 ml of enzyme preparation; dicumarol dissolved in 0.15 ml of a dilute alcoholic KOH solution. After 8 minutes pre-incubation, NADPH (4.9 μmoles) dissolved in 0.15 ml of Tris-MgCl$_2$ buffer was added. Solid ammonium sulfate to give 20% saturation was added after 90 seconds incubation and cytochromes P-450 and P-420 were isolated and measured as described in the methods.

Control (-----); plus dicumarol, 23 (······) or 116 (-----) μmoles/l, final concentration.
Fig. 45. Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of low concentrations of dicumarol.

Incubations were carried out as described in Fig. 44. Cytochrome P-450 (o), cytochrome P-420 (©).
leads to an increased recovery of cytochrome P-450, 11β-hydroxylation is still inhibited (Figs. 37, 38 and 39).

As the dicumarol concentration is increased above 23 μmoles/l the cytochrome P-450 content of the isolated 20-40% ammonium sulfate fractions decreases and cytochrome P-420 correspondingly increases (Fig. 46). At a dicumarol concentration of 145 μmoles/l, all cytochrome P-450 originally present in the enzyme preparation has been removed. The increase in cytochrome P-420 levels accompanying the decrease in cytochrome P-450 indicates that the breakdown of cytochrome P-450 produced by dicumarol results in the formation of cytochrome P-420. Thus dicumarol, at these concentrations, appears to alter the hydrophobic environment of cytochrome P-450, converting it to cytochrome P-420.

In an attempt to determine whether the two effects of dicumarol on cytochrome P-450 are related to its inhibition of 11β-hydroxylation, cytochrome P-450 levels were measured after incubation in the presence of deoxycorticosterone and/or dicumarol (Fig. 47). In the absence of dicumarol, the addition of deoxycorticosterone (7 or 34 μmoles/l) produces an increase in cytochrome P-450. The increased cytochrome P-450 content in the presence of deoxycorticosterone has been previously demonstrated (Fig. 17) and at a concentration of 34 μmoles/l the deoxycorticosterone effect is maximal. The addition of dicumarol (23 μmoles/l) produces a further increase in cytochrome P-450. The extent of this increase in cytochrome P-450 is essentially the same in the presence of deoxycorticosterone
Fig. 46. Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of high concentrations of dicumarol.

Incubations were carried out as described in Fig. 44. Cytochrome P-450 (o), cytochrome P-420 (©).
Fig. 47. Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of dicumarol and deoxycorticosterone.

Incubations were carried out as described in Fig. 44. Deoxycorticosterone was added dissolved in 0.15 ml of propylene glycol.

Deoxycorticosterone final concentration (μmoles/l): 0 (○), 7.4 (Δ), 34 (○).
(7 or 34 μmoles/l) or in its absence. This indicates that the effects of dicumarol and deoxycorticosterone are independent. Furthermore, if these effects are produced by these compounds binding to cytochrome P-450 then they must bind at different sites on the hemoprotein. This finding is compatible with the noncompetitive nature of inhibition of 11β-hydroxylation afforded by dicumarol at this concentration (Figs. 38 and 39) The additive effects of dicumarol and deoxycorticosterone on cytochrome P-450 also reveal that the increase produced by either compound alone is not the maximal effect possible on cytochrome P-450.

As the dicumarol concentration is increased above 60 μmoles/l, there is a decrease in cytochrome P-450 at both deoxycorticosterone concentrations (7 and 34 μmoles/l) studied (Fig. 47). The dicumarol concentration that initiates a decrease in cytochrome P-450 is higher (above 60 μmoles/l) in the presence of deoxycorticosterone than in the absence (above 23 μmoles/l) of this steroid. The general profile of the loss of cytochrome P-450 is the same in both the presence and absence of deoxycorticosterone, but at any given dicumarol concentration, the presence of deoxycorticosterone increases the amount of cytochrome P-450 that can be recovered. Thus it appears that the decomposition of cytochrome P-450 produced by dicumarol competes with the stabilization of cytochrome P-450 afforded by the steroid substrate.

The data on cytochrome P-450 (Fig. 47) were compared to the kinetic data on dicumarol inhibition previously determined
(Fig. 39) in order to ascertain whether the biphasic nature of the kinetic curves were related to the effects of dicumarol on cytochrome P-450. If the decomposition of cytochrome P-450 is responsible for the second dicumarol inhibitory action on 11β-hydroxylation, then this decomposition would significantly alter the overall reaction only when cytochrome P-450 levels had fallen below those levels observed in the absence of dicumarol but in the presence of deoxycorticosterone. Examination of the data in Figure 47 reveals that in the presence of deoxycorticosterone (7 µmoles/l), cytochrome P-450 levels decrease to less than that of the control at a dicumarol concentration above 95 µmoles/l, while at a deoxycorticosterone concentration of 34 µmoles/l this value is 100 µmoles/l. Examination of the kinetic data in Figure 39 reveals that at a deoxycorticosterone concentration of 7 µmoles/l the second inhibitory action of dicumarol is first apparent at a dicumarol concentration of about 105 µmoles/l, and at a deoxycorticosterone concentration of 35 µmoles/l this value is 135 µmoles/l. Thus there is a correlation between dicumarol concentrations bringing about the decomposition of cytochrome P-450 to levels lower than that of incubations containing steroid alone, and the dicumarol concentrations at which the secondary inhibitory effect on 11β-hydroxylation is demonstrable. Both the decomposition of cytochrome P-450 and the inhibition of 11β-hydroxylation produced at high dicumarol concentrations are sensitive to changes in substrate deoxycorticosterone concentration suggesting that the two events are related. The inhibition of
llβ-hydroxylation at high dicumarol concentrations is a result of decomposition of cytochrome P-450.

A time study on the decomposition of cytochrome P-450 in the presence of dicumarol (23 or 233 μmoles/l) was carried out to determine whether the inhibitor was affecting the rate of decomposition of this hemoprotein. The results are shown in Figure 48. Earlier studies had shown that the rate of breakdown of cytochrome P-450 was retarded in the presence of deoxycorticosterone (Fig. 20). This result is confirmed by the data presented in Figure 48. The presence of deoxycorticosterone reduces the rate of P-450 disappearance by 50%. At the lower concentration (23 μmoles/l) dicumarol decreases the rate of P-450 breakdown by 19%. This decrease in the rate of breakdown of cytochrome P-450 produced by dicumarol is not as great as that produced in the presence of substrate deoxycorticosterone. At high concentration (233 μmoles/l) dicumarol has an opposite effect to that observed at lower concentrations. The rate of cytochrome P-450 breakdown is enhanced by 47% over the control rate.

The results shown in Figure 48 permit further explanation of the cytochrome P-450 levels illustrated in Figure 47. The cytochrome P-450 levels were measured after approximately 10 minutes of incubation at 37° C and are a result of the effects of deoxycorticosterone and dicumarol on the rate of cytochrome P-450 decomposition. Dicumarol at low concentrations alters cytochrome P-450 in some manner which increases its stability. This effect is analogous to the deoxycorticosterone
Fig. 48. Effect of deoxycorticosterone and dicumarol on the rate of cytochrome P-450 disappearance.

Appropriate reaction flasks contained: deoxycorticosterone dissolved in 0.15 ml of propylene glycol; 2.1 ml of Tris-MgCl₂ buffer, 3.9 ml of enzyme preparation; and dicumarol dissolved in 0.15 ml of a dilute alcoholic KOH solution. After 0, 4, 8, or 12 minutes of incubation solid ammonium sulfate to give 20% saturation was added and cytochrome P-450 then was isolated and measured as described in the methods.

Control (○); plus deoxycorticosterone, final concentration, 39 µmoles/1 (○); plus dicumarol final concentration 23 (○), or 233 (▲) µmoles/1.
sterone stabilization of cytochrome P-450 but is not as pronounced. Dicumarol at high concentration, on the other hand, alters cytochrome P-450 in a manner resulting in an increased instability and rapid degradation to cytochrome P-420. The distinct effects of dicumarol may indicate that this inhibitor can bind to more than one site on cytochrome P-450, a finding in agreement with the biphasic inhibition kinetics previously observed (Fig. 39).

The effects of dicumarol on cytochrome P-450 and 11β-hydroxylation were also studied employing a lyophilized enzyme preparation from sonicated mitochondria. This preparation has the advantage that cytochrome P-450 measurements can be made directly on the incubation mixture, eliminating the necessity for ammonium sulfate fractionation. Moreover, cytochrome P-450 that has been enzymatically reduced with NADPH can be measured directly. The effects of dicumarol on NADPH-reduced cytochrome P-450 may then be compared with those in the 20-40% ammonium sulfate fraction with dithionite-reduced cytochrome P-450. The results are shown in Figure 49.

At a dicumarol concentration of 112 μmoles/l there is a slight decrease in the absorption maximum at 450 μm with a corresponding increase in absorption at 420 μm. When the dicumarol concentration is increased to 224 μmoles/l the absorption maximum at 450 μm is decreased by 50%. This is accompanied by a large increase in absorption at 420 μm. The spectral changes produced by dicumarol indicate that cytochrome P-450
Fig. 49. Effect of dicumarol on cytochrome P-450 in the 11β-hydroxylase prepared from sonicated adrenal mitochondria.

Each cuvette contained: 0.05 ml of propylene glycol; 1.0 ml of Tris-MgCl₂ buffer; 25 mg of lyophilized enzyme preparation dissolved in 1.95 ml of a 0.154 M solution of KCl; and 0, 0.35, or 0.70 μmole of dicumarol dissolved in 0.07 ml of a dilute alcoholic KOH solution. After 8 minutes pre-incubation at 37° C, NADPH (1.1 μmoles) was added dissolved in 0.05 ml of Tris-MgCl₂ buffer to both the sample and reference cuvettes and the carbon monoxide difference spectrum was determined.

Control (———); plus dicumarol 112 (••••), or 224 (-----) μmoles/l, final concentration.
in the sonicated enzyme preparation is converted to cytochrome P-420 in the presence of dicumarol, an effect also observed with the mitochondrial acetone powder preparation (Fig. 44). Similar results to those shown in Figure 49 are obtained if cytochrome P-450 is estimated by chemical reduction with dithionite. The effect of dicumarol on cytochrome P-450 in the sonicated mitochondria preparation is less than its effect on the cytochrome P-450 in the acetone powder preparation. At a dicumarol concentration of 224 μmoles/l, the extent of conversion of cytochrome P-450 to cytochrome P-420 is 50% in the former preparation (Fig. 49) but 100% in the latter (Fig. 46).

The effects of various dicumarol concentrations on cytochromes P-450, P-420 and 11β-hydroxylation in the lyophilized enzyme preparation are shown in Table XII. Both 11β-hydroxylation of deoxycorticosterone and the cytochrome P-450 content diminish as the concentration of dicumarol is increased. The decrease in cytochrome P-450 is accompanied by an increase in cytochrome P-420. However there is strict correlation between cytochrome P-450 and 11β-hydroxylation only at the highest dicumarol concentration tested (233 μmoles/l) when both 11β-hydroxylase activity and cytochrome P-450 have been reduced by 50%. This may indicate that at intermediate dicumarol concentrations, the effect of the inhibitor on 11β-hydroxylation may not be wholly ascribed to the breakdown of cytochrome P-450. Dicumarol does not produce as extensive an inhibition of 11β-hydroxylation in the sonicated mitochondrial
TABLE XII

Effect of dicumarol on the 11β-hydroxylase activity, cytochrome P-450, and cytochrome P-420 content of the enzyme system prepared by sonication of adrenal mitochondria

<table>
<thead>
<tr>
<th>Dicumarol concentration (µmoles/l)</th>
<th>Percent of control 11β-hydroxylation</th>
<th>Percent of control cytochrome P-450</th>
<th>Percent of control cytochrome P-420</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.3</td>
<td>95.5</td>
<td>100</td>
<td>110</td>
</tr>
<tr>
<td>58.1</td>
<td>92.3</td>
<td>88</td>
<td>120</td>
</tr>
<tr>
<td>116</td>
<td>68.8</td>
<td>83</td>
<td>190</td>
</tr>
<tr>
<td>174</td>
<td>58.3</td>
<td>67</td>
<td>320</td>
</tr>
<tr>
<td>233</td>
<td>51.4</td>
<td>50</td>
<td>330</td>
</tr>
</tbody>
</table>

Cytochromes P-450 and P-420 were measured in incubations carried out as described in Fig. 49. For the measurement of 11β-hydroxylase activity each reaction flask contained: 0.094 µmole of deoxycorticosterone-1,2-³H (specific activity 1.89 x 10⁷ cpm/µmole) dissolved in 0.05 ml of propylene glycol; 0.70 ml of Tris-MgCl₂ buffer; 17 mg of lyophilized enzyme powder dissolved in 1.30 ml of a 0.154 M KCl solution; dicumarol dissolved in 0.05 ml of a dilute alcoholic KOH solution. After 8 minutes preincubation, NADPH (1.7 µmoles) dissolved in 0.05 ml of Tris-MgCl₂ buffer was added. A sample (1.0 ml) was removed from each flask after 90 seconds of incubation and pipetted into ethyl acetate (2 ml) to terminate the reaction.
preparation (Table XII) as it does in the acetone powder preparation (Fig. 37). Moreover, no increase in cytochrome P-450 is observed at low dicumarol concentrations in the sonicated mitochondria preparation (Table XII). This finding, as well as the decreased sensitivity of cytochrome P-450 and 11β-hydroxylation to higher dicumarol concentrations in the sonicated preparation when compared to the acetone powder preparation, can be best explained by a greater stability of the hemoprotein in the sonicated preparation. It has been previously noted that cytochrome P-450 of the sonicated preparation was stable under incubation conditions resulting in a 64% decrease of this hemoprotein in the acetone powder preparation. Thus any stabilizing effect of dicumarol at low concentrations would not be evident, and cytochrome P-450 in the sonicated enzyme preparation would be more resistant to decomposition at high dicumarol concentrations.

(b) Effect of Metopirone on Cytochromes P-450 and P-420

The kinetics of Metopirone inhibition of 11β-hydroxylation are different from those observed with dicumarol. Studies of the effect of Metopirone on cytochrome P-450 were therefore undertaken to determine whether Metopirone also affected this hemoprotein in a manner different from dicumarol.

Previous studies revealed that Metopirone inhibited 11β-hydroxylation at low concentrations (Fig. 41) and produced a stimulation of NADPH oxidation at high concentrations (Fig. 40). The effect of a wide range of Metopirone concentrations
on cytochrome P-450 was therefore examined. The results are shown in Figure 50. At low concentrations, Metopirone produces a large increase in the cytochrome P-450 recovered after 8 minutes of incubation. This effect of Metopirone is similar to that observed with deoxycorticosterone (Fig. 17) and may reflect the competitive nature of Metopirone inhibition of 11β-hydroxylation. As Metopirone concentrations increase, there is a decrease in the amount of recovered cytochrome P-450 (Fig. 50) to a level that is not influenced by further increases in Metopirone concentration. Figure 50 also shows the cytochrome P-420 levels at various Metopirone concentrations after 8 minutes of incubation at 37°C. At low concentrations of Metopirone, cytochrome P-420 decreases, perhaps due to the increased cytochrome P-450 present at these inhibitor concentrations. As the Metopirone concentration is increased, cytochrome P-420 levels remain low and constant.

A time study of the rate of decomposition of cytochrome P-450 in the presence of Metopirone was carried out. Metopirone concentrations in the region of increased cytochrome P-450 recovery (3.5 μmoles/l) and decreased cytochrome P-450 recovery (27.2 μmoles/l) as indicated in Figure 50 were selected for the time studies. The results are illustrated in Figure 51. The addition of a small quantity of Metopirone (3.5 μmoles/l, final concentration) prevents the time-dependent loss of cytochrome P-450 observed in the absence of this inhibitor (Fig. 51a). Cytochrome P-450 levels are higher in the presence of Metopirone than in its absence at
Fig. 50. Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of Metopirone.

Each reaction flask contained: 0.15 ml of propylene glycol; 2.1 ml of Tris-MgCl₂ buffer; 3.9 ml of enzyme preparation; and Metopirone dissolved in 0.15 ml of Tris-MgCl₂ buffer. Solid ammonium sulfate to give 20% saturation was added after 8 minutes of incubation and cytochromes P-450 and P-420 were isolated and measured as described in the methods.

Cytochrome P-450 (○), cytochrome P-420 (△).
Fig. 51. Effect of Metopirone on the rate of cytochrome P-450 disappearance.

Incubations were carried out as described in Fig. 50. Solid ammonium sulfate was added after 0, 4, 8, or 12 minutes of incubation and cytochrome P-450 was isolated and measured as described in the methods.

Fig. 51a. Control (○); plus Metopirone, 3.5 µmoles/l, final concentration (○).
Fig. 51b. Control (Δ); plus Metopirone, 28 µmoles/l, final concentration (Δ).
all incubation times. Therefore Metopirone may be altering cytochrome P-450 to prevent its spontaneous decomposition either by inducing an alteration in the hemoprotein to a more stable conformation, or by binding to the cytochrome and thus protecting a labile group. The effect of Metopirone at this concentration is very similar to that observed with deoxycorticosterone (Fig. 20). At the higher Metopirone concentration (27.2 μmoles/l) less cytochrome P-450 is recovered at zero time in the presence of Metopirone than in its absence (Fig. 51b). The lower level of cytochrome P-450 remains unaltered throughout the incubation period. Thus the cytochrome P-450 levels in the control incubation (no Metopirone) are higher than those in the presence of Metopirone during the early stages of incubation, but fall below those in the presence of Metopirone at the later stages of incubation, due to the continual degradation of cytochrome P-450 throughout the incubation period. At high Metopirone concentrations the level of cytochrome P-450 is not affected by either incubation time (Fig. 51b) or by changes in Metopirone concentration in this region (Fig. 50). Therefore the addition of large amounts of Metopirone may produce a further alteration in cytochrome P-450 to a second stable form. The initial loss of hemoprotein may indicate that during the course of this alteration there is a very rapid loss of a portion of the cytochrome P-450. Alternatively, the loss of absorbance at 450 μm may be attributed to an alteration of cytochrome P-450 that results in a decrease in intensity of the absorption maximum at this wavelenth.
Because the 11β-hydroxylase inhibition by Metopirone is associated with low concentrations of this compound (Figs. 41 and 43), the lower limit of Metopirone protection of cytochrome P-450 was investigated. The results are shown in Figure 52. A maximal increase in cytochrome P-450 recovery is observed at a Metopirone concentration of 0.5 μmole/l and a significant increase at 0.05 μmole/l. Thus Metopirone can alter this cytochrome at concentrations in the region of its $K_i$ (0.1 μmole/l) for 11β-hydroxylase inhibition. This suggests that the effect of Metopirone on cytochrome P-450 is related to its inhibitory action on 11β-hydroxylation. No protection of cytochrome P-420 is afforded by Metopirone at these concentrations (Fig. 52).

The effect of Metopirone on cytochrome P-450 in the presence of NADPH was next examined. Duplicate reaction mixtures containing Metopirone at concentrations of 0, 1, 5 or 20 μmoles/l were pre-incubated for 8 minutes at 37° C. To one series of flasks NADPH was added. All flasks were incubated at 37° C for an additional 90 seconds and, in each instance, the cytochrome P-450 fraction was isolated. The addition of NADPH (Fig. 53) does not significantly influence the effect of Metopirone on cytochrome P-450. Similar results had been found previously with regard to the effect of NADPH on deoxycorticosterone protection of cytochrome P-450.

The inter-relationship between the effects of substrate deoxycorticosterone and Metopirone on cytochrome P-450 was ascertained by the simultaneous addition of the two compounds
Fig. 52. Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of low concentrations of Metopirone.

Incubations were carried out as described in Fig. 50. Cytochrome P-450 (o), cytochrome P-420 (e).
Fig. 53. Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of Metopirone and NADPH.

Incubations were carried out as described in Fig. 50. After pre-incubation for 8 minutes, NADPH (3 μmoles) dissolved in 0.15 ml of Tris-MgCl₂ buffer was added to one series of reaction flasks, 0.15 ml of Tris-MgCl₂ buffer was added to the other. Solid ammonium sulfate was added to each reaction flask after 90 seconds of incubation and cytochrome P-450 then was isolated and measured as described in the methods. Control (o), plus NADPH (●).
to the reaction mixture. The effects of increasing Metopirone concentrations on cytochrome P-450 in both the absence and presence of deoxycorticosterone are shown in Figure 54. Both deoxycorticosterone (Fig. 17) and low concentrations of Metopirone (Fig. 52) produce an increase in cytochrome P-450. These effects are also demonstrated in Fig. 54 when deoxycorticosterone is added (no Metopirone) or when Metopirone is added (1 μmole/l, no deoxycorticosterone). However simultaneous addition of deoxycorticosterone (42 μmoles/l) and Metopirone (1 μmole/l) does not produce an increase in cytochrome P-450 greater than that produced by Metopirone alone. Thus the two effects are not additive and differ from those observed on addition of deoxycorticosterone and low concentrations of dicumarol (Fig. 47). Metopirone can eliminate the effect of deoxycorticosterone on cytochrome P-450 suggesting that the inhibitor prevents the binding of steroid substrate. This finding concurs with the competitive nature of Metopirone inhibition of 11β-hydroxylation (Fig. 43). The cytochrome P-450 level is increased to a greater extent in the presence of Metopirone alone (1 μmole/l) than in the presence of deoxycorticosterone alone (42 μmoles/l), a finding that may reflect the extremely high affinity of Metopirone for the 11β-hydroxylase (K_i Metopirone = 1.0 x 10^{-7} moles/l; K_m deoxycorticosterone = 5.5 x 10^{-6} moles/l). Deoxycorticosterone obviates the decrease in cytochrome P-450 that is observed at higher Metopirone concentrations (Fig. 54). If the effect of high concentrations of Metopirone on cytochrome P-450 reflects the
Fig. 54. Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of Metopirone and deoxycorticosterone.

Incubations were carried out as described in Fig. 50. Deoxycorticosterone (42 μmoles/l, final concentration) was added dissolved in propylene glycol (0.15 ml). Metopirone (o), Metopirone and deoxycorticosterone (o).
binding of Metopirone to a second site on the hemoprotein, then it must be concluded that deoxycorticosterone can prevent the binding of Metopirone to this second site.

The effect of Metopirone on cytochrome P-450 in the sonicated enzyme preparation was examined. The results are shown in Table XIII. At no concentration does Metopirone alter the amount of NADPH-reducible cytochrome P-450, indicating a difference in the stability of cytochrome P-450 in the sonicated and acetone powder enzyme preparations. As the cytochrome P-450 measured in this experiment (Table XIII) was reduced enzymatically by NADPH, one may conclude that Metopirone does not affect the reduction of cytochrome P-450 by NADPH.

(c) Substrate and Inhibitor-Induced Difference Spectra of Cytochrome P-450

Recent studies have revealed that the addition of substrates to both the microsomal and mitochondrial hydroxylase systems produce spectral changes that can be measured by the technique of difference spectrophotometry (86,87,95-98). Evidence indicates that this spectrum is the result of interaction and binding of the substrate with cytochrome P-450 (98) supporting the proposition that cytochrome P-450 is the steroid-activating as well as the oxygen-activating component of steroid hydroxylase systems. The ability of hydroxylase substrates to induce spectral changes was employed to study the interaction of deoxycorticosterone, dicumarol, and Metopirone with cytochrome
TABLE XIII

Effect of Metopirone on cytochromes P-450 and P-420 in the enzyme system prepared by sonication of adrenal mitochondria

<table>
<thead>
<tr>
<th>Metopirone concentration (µmoles/l)</th>
<th>Δ Absorbance 450-500 mµ (P-450)</th>
<th>Δ Absorbance 420-500 mµ (P-420)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.030</td>
<td>0.007</td>
</tr>
<tr>
<td>0</td>
<td>0.032</td>
<td>0.009</td>
</tr>
<tr>
<td>3.4</td>
<td>0.029</td>
<td>0.018</td>
</tr>
<tr>
<td>6.8</td>
<td>0.033</td>
<td>0.014</td>
</tr>
<tr>
<td>6.8</td>
<td>0.031</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Each cuvette contained: 0.02 ml of propylene glycol; 0.33 ml of Tris-MgCl₂ buffer; 9 mg of lyophilized enzyme powder dissolved in 0.65 ml of a 0.154 M solution of KCl; and Metopirone dissolved in 0.02 ml of Tris-MgCl₂ buffer. After 8 minutes pre-incubation at 37° C, NADPH (0.28 µmole) dissolved in 0.02 ml of Tris-MgCl₂ buffer was added to both the sample and reference cuvettes. The carbon monoxide difference spectrum was then determined.
P-450.

(i) Substrate-induced difference spectrum of cytochrome P-450.

Because the spectral changes induced by steroid substrates are a result of interaction between the substrate and cytochrome P-450, the enzyme fraction precipitating between 20% and 40% saturation with ammonium sulfate was employed in these studies. This fraction has been shown previously to contain both cytochrome P-450 and cytochrome P-420 (Fig. 10b). In addition, the cytochrome P-450 in this fraction is relatively stable so that the interaction of deoxycorticosterone, Metopirone and dicumarol with cytochrome P-450 could be measured spectrophotometrically without having to consider that any effect of these compounds was due to stabilization or decomposition of cytochrome P-450. As a precaution, spectra were measured at room temperature immediately after addition of the various agents.

The deoxycorticosterone-induced difference spectrum of cytochrome P-450 as a function of the amount of original enzyme solution used to prepare the 20-40% ammonium sulfate fraction is shown in Figure 55. The spectrum has a trough at 420 m\(\mu\) and a broad maximum centered at 385 m\(\mu\). A similar difference spectrum with deoxycorticosterone has been observed by Cooper et al. (86) with an adrenal mitochondrial preparation. In addition, several substrates of the microsomal hydroxylase system induce similar spectra in adrenal and liver microsomes (85,87,95-98).
Fig. 55. Spectral changes produced by addition of deoxycorticosterone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

The 20-40% ammonium sulfate fraction from 2, 4, or 8 ml of enzyme preparation was dissolved in 1.6 ml of a solution containing Tris-MgCl₂ buffer (0.6 ml) and 0.154 M KCl (1.0 ml) and divided equally between two cuvettes. After recording the baseline (500-375 μm), deoxycorticosterone (84 μmoles/l final concentration) was added dissolved in 0.02 ml of propylene glycol to the sample cuvette. The reference cuvette received 0.02 ml of propylene glycol. The difference spectrum was then recorded.

20-40% ammonium sulfate fraction from: 2 (----), 4 (······), and 8 (-----) ml of enzyme preparation.
The effect of varying deoxycorticosterone concentrations on the induced spectral change is shown in Figure 56. The intensities of both the 420 m\(\mu\) trough and the 385 m\(\mu\) peak are proportional to the steroid concentration. A plot of the change of absorbance (420-403 m\(\mu\) or 385-420 m\(\mu\)) as a function of steroid concentration gives the normal type of enzyme-substrate saturation curve (Fig. 57). From the Lineweaver-Burk plot of these data the substrate concentration required for half-maximal spectral change can be obtained (Fig. 57). This value has been termed the "spectral dissociation constant" by Schenkman \textit{et al.} (97) and is a measure of the enzyme-substrate complex dissociation. The value obtained for the deoxycorticosterone-cytochrome P-450 complex is 7 \(\mu\)moles/l, a value similar to the \(K_m\) for 11\(\beta\)-hydroxylation, suggesting a close relationship between the spectral change observed upon interaction of deoxycorticosterone and cytochrome P-450, and the formation of corticosterone from deoxycorticosterone. The same value for the spectral dissociation constant is obtained using either \(\Delta\) absorbance (420-403) or \(\Delta\) absorbance (385-420), indicating that the overall difference spectrum as well as the 420 m\(\mu\) trough is a measure of deoxycorticosterone binding to the enzyme.

(ii) Effect of dicumarol on the substrate-induced difference spectrum of cytochrome P-450.

Direct interaction of dicumarol with cytochrome P-450 could not be measured because dicumarol itself absorbed in
Fig. 56. The effect of deoxycorticosterone concentration on the magnitude of the spectral change produced by addition of deoxycorticosterone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

Incubations were carried out as described in Fig. 55. The 20-40% ammonium sulfate fraction from 8 ml of enzyme preparation was dissolved in 1.6 ml of the Tris-KCl mixture and divided equally between two cuvettes. Deoxycorticosterone was added to the sample cuvette dissolved in 0.02 ml of propylene glycol.

The final concentration (umoles/l) of deoxycorticosterone was: 2.44 (......); 5.49 (-----); 12.2 (----); or 21.8 (-----).
Fig. 57. Double reciprocal plot of the change in absorbance (385-420 μm) or (420-403 μm) as a function of deoxycorticosterone concentration.

Incubations were carried out as described in Fig. 56. Reciprocal of absorbance calculated from the change of absorbance from: 385 to 420 μm (o), 403 to 420 (e).
the region of the spectrum (500-375 μm) employed to measure these interactions. However the effect of dicumarol on the substrate difference spectrum was examined.

Figure 58 shows the effect of dicumarol at concentrations of 176 and 223 μmoles/1 on the spectral changes produced by deoxycorticosterone at concentrations of 7.8 μmoles/1 (Fig. 58a) and 39 μmoles/1 (Fig. 58b). At both concentrations dicumarol decreases the intensity of both the 420 μm trough and the 385 μm peak, the decrease being proportional to the amount of dicumarol present. The influence of dicumarol on the difference spectrum is diminished by an increase in the substrate concentration. The results suggest that dicumarol either interferes with the binding of deoxycorticosterone to cytochrome P-450, or interferes with the alteration of the hemoprotein by deoxycorticosterone that produces these spectral changes. The cytochrome P-450 content of each of the incubation mixtures employed to measure these spectral changes was determined. In all instances, the hemoprotein content was the same, indicating that dicumarol was not affecting the deoxycorticosterone-induced spectral changes by causing a degradation of cytochrome P-450.

The ability of deoxycorticosterone to induce spectral changes in cytochrome P-450 in the presence of increasing dicumarol concentrations is shown in Figure 59. Deoxycorticosterone at concentrations similar to those employed in the inhibition kinetic studies (Fig. 39), was added to reaction mixtures containing dicumarol at concentrations ranging from
The 20-40% ammonium sulfate fraction obtained from 8 ml of enzyme preparation was dissolved in a solution made up of 0.56 ml of Tris-MgCl₂ buffer and 1.0 ml of 0.154 M KCl. Dicumarol dissolved in 0.04 ml of a dilute alcoholic KOH solution was added and the mixture was equally divided between two cuvettes. A baseline was recorded. Deoxycorticosterone dissolved in 0.02 ml of propylene glycol was added to the sample cuvette; the reference cuvette received 0.02 ml of propylene glycol. The difference spectrum was then recorded.

Fig. 58a. Deoxycorticosterone, final concentration, 7.8 μmoles/l. Dicumarol, final concentration, μmoles/l: 0 (-----), 176 (------), 233 (••••).

Fig. 58b. Deoxycorticosterone, final concentration, 39 μmoles/l. Dicumarol, final concentration, μmoles/l: 0 (-----), 176 (------), 233 (••••).
Fig. 59. Effect of dicumarol on the magnitude of the spectral change (385-420 m\(\mu\)) produced by addition of deoxycorticosterone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

Incubations were carried out as described in Fig. 58. Deoxycorticosterone, final concentration, \(\mu\)moles/l: 7.8 (\(\bullet\)), 39 (\(\circ\)).
23 to 233 μmoles/l. It is apparent that little effect on the intensity of the spectral change produced at either deoxycorticosterone concentration (7.8 or 39 μmoles/l) is observed at dicumarol concentrations below 100 μmoles/l, the range over which dicumarol is a noncompetitive inhibitor of 11ß-hydroxylation (Figs. 38 and 39). At dicumarol concentrations greater than 100 μmoles/l there is a decrease in the magnitude of the spectral change induced by deoxycorticosterone at a concentration of 7.8 μmoles/l; a 50% decrease is observed at a dicumarol concentration of 233 μmoles/l. This effect of dicumarol is not so prominent at the higher concentration of steroid substrate (39 μmoles/l) indicating that an increase in deoxycorticosterone concentration can overcome the inhibitory effect of dicumarol. The influence of dicumarol on these deoxycorticosterone-induced spectra cannot wholly account for the degree of inhibition of 11ß-hydroxylation by dicumarol at high concentrations (Fig. 39). At a deoxycorticosterone concentration of 7.0 μmoles/l, 11ß-hydroxylation is inhibited 79% while the spectral change is inhibited 50%. At a deoxycorticosterone concentration of 35 μmoles/l, 11ß-hydroxylation is inhibited 62% while the spectral change is inhibited approximately 20%.

Only high concentrations of dicumarol (above 100 μmoles/l) affect the deoxycorticosterone-induced difference spectrum. In addition the effect of dicumarol can be reduced considerably by an increase in substrate concentration. Therefore it is likely that the effect of dicumarol on the substrate-induced
difference spectrum of cytochrome P-450 is related to the second inhibitory action of dicumarol on 11β-hydroxylation that is observed at high concentrations of this compound (Fig. 39).

(iii) Metopirone-induced difference spectrum of cytochrome P-450.

Unlike dicumarol, Metopirone does not exhibit absorption in the region of the spectrum where the deoxycorticosterone effects on cytochrome P-450 are measured. Therefore if there is a direct interaction between Metopirone and cytochrome P-450, this could be studied spectrophotometrically. That such an interaction does occur is demonstrated by the Metopirone-induced spectral changes shown in Figure 60. The difference spectrum exhibits an absorption maximum at 422 μm and a broad trough centered around 400 μm. The concentration of Metopirone required to induce a significant spectral change is greater than that required to inhibit 11β-hydroxylation. Therefore the Metopirone-induced spectral change is likely related to the secondary effect on cytochrome P-450 produced by high Metopirone concentrations previously noted (Fig. 50), and may also be related to the increased rate of NADPH oxidation that is observed at high Metopirone concentrations (Fig. 40). The spectral change induced by Metopirone (Fig. 60) is similar to those produced by organic solvents in microsomal preparations described by Imai and Sato (98). They observed that organic solvents such as methanol, ethanol, and 2-propanol, at concentrations from 3% to 5%, produced difference spectra with an
Fig. 60. Spectral changes produced by addition of Metopirone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

The 20-40% ammonium sulfate fraction obtained from 8 ml of the enzyme preparation was dissolved in a solution made up of 0.56 ml of Tris-MgCl₂ buffer and 1.0 ml of 0.154 M KCl. 0.04 ml of propylene glycol was added and the mixture was divided equally between two cuvettes. A baseline (500-375 μm) was recorded. Metopirone, dissolved in 0.01 ml of Tris-MgCl₂ buffer was added to the sample cuvette; the reference cuvette received 0.01 ml of Tris-MgCl₂ buffer. The difference spectrum was then recorded.

Metopirone final concentration, μmoles/l: 0.1 (-----), 1.0 (·····), 20 (--•--), 200 (-----), 400 (———).
absorption maximum at 420 m\(\mu\) and a trough at 390 m\(\mu\). No conversion of cytochrome P-450 to cytochrome P-420 took place in these experiments. The solvent concentrations producing these difference spectrum were of the same order of magnitude as the concentrations causing changes in protein conformation through a disturbance of hydrophobic interactions. It is quite likely that the spectral changes produced at high Metopirone concentrations are a result of a similar effect.

(iv) The effect of Metopirone on the substrate-induced difference spectrum of cytochrome P-450.

The effect of Metopirone on the substrate deoxycorticosterone-induced difference spectrum is shown in Figure 61. The presence of Metopirone at a concentration of 0.1 or 0.2 \(\mu\)mole/\(l\) results in a diminution of the spectrum in a manner similar to that produced by dicumarol. However, Metopirone inhibits the substrate difference spectrum at much lower concentrations than does dicumarol (Fig. 58), and at concentrations lower than those required for Metopirone alone elicit spectral changes (Fig. 60). The concentrations of Metopirone that decrease the substrate difference spectrum are the same as those that produce an inhibition of 11\(\beta\)-hydroxylation (Fig. 43). The effect of increasing substrate concentration is shown in Figure 61b and Figure 62. The inhibition of the deoxycorticosterone difference spectrum by Metopirone can be overcome by an increase in steroid substrate concentration. This indicates a competition of these two compounds for binding to
Fig. 61. Effect of Metopirone on the spectral changes produced by addition of deoxycorticosterone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

The 20-40% ammonium sulfate fraction obtained from 8 ml of enzyme preparation was dissolved in a solution made up of 0.56 ml of Tris-MgCl₂ buffer and 1.0 ml of 0.154 M KCl and the mixture was equally divided between two cuvettes. Deoxycorticosterone dissolved in 0.02 ml of propylene glycol was added and the difference spectrum was determined as described in Fig. 55. Metopirone dissolved in 0.01 ml of Tris-MgCl₂ buffer then was added to both the sample and reference cuvettes and the difference spectrum was again recorded.

Fig. 61a. Deoxycorticosterone final concentration, 2.4 µmoles/l, Metopirone final concentration, µmole/l: 0 (——), 0.1 (-----), 0.2 (······).

Fig. 61b. Deoxycorticosterone final concentration, 21.8 µmoles/l, Metopirone final concentration, µmole/l: 0 (——), 0.1 (-----), 0.2 (······).
Fig. 62. Effect of Metopirone on the magnitude of the spectral change (385-420 μμ) produced by addition of deoxycorticosterone to the 20-40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation.

Incubations were carried out as described in Fig. 61. Metopirone final concentration, μmole/l: 0 (○), 0.1 (□), 0.2 (□).
cytochrome P-450, a finding in agreement with the competitive nature of Metopirone inhibition of 11β-hydroxylation (Fig. 43). The potent inhibitory action of Metopirone is apparent however, for significant inhibition of the deoxycorticosterone-induced difference spectrum by Metopirone at a concentration of 0.2 μmole/l still persists at the highest deoxycorticosterone concentration (Fig. 62). These data suggest that interaction of either Metopirone or deoxycorticosterone with cytochrome P-450 interferes with the binding of the other to this hemoprotein, and that cytochrome P-450 has a greater affinity for Metopirone than for deoxycorticosterone.

DISCUSSION

The experiments that have been described suggest that the inhibition of 11β-hydroxylation by dicumarol and Metopirone is related to their effects on cytochrome P-450 and on the interaction of steroid substrate with this hemoprotein.

Various groups of workers (85-87,95-98) have demonstrated that addition of substrates of both microsomal and mitochondrial hydroxylases to preparations of these systems causes spectral changes in the soret region of the spectrum characteristic of hemoproteins. Imai and Sato have provided evidence suggesting that the spectral changes are due to substrate interaction with cytochrome P-450 (98), drawing attention to the role of this hemoprotein as the substrate-activating site in the hydroxylation process. We have demonstrated that deoxycorticosterone can produce a spectral change in cytochrome P-450
isolated from an adrenal mitochondrial acetone powder. This spectral change is dependent on the amount of both substrate and enzyme present in the reaction mixture. The similarity of the "spectral dissociation constant" and the $K_m$ for $11\beta$-hydroxylation of substrate deoxycorticosterone suggests that the observed spectral change in the presence of substrate is related to the hydroxylation process and that it is a measure of the formation of an enzyme-substrate complex.

The precise mechanism whereby deoxycorticosterone, and other hydroxylase substrates, induce spectral changes in cytochrome P-450 is not known. A reasonable hypothesis would be that the substrate binds to a specific site on the protein moiety of cytochrome P-450 causing a conformational alteration in the hemoprotein molecule with an accompanying modification of ligand interaction with the heme iron of cytochrome P-450. Data obtained by electron paramagnetic resonance spectroscopy suggests that substrate binding does modify ligand interaction with the heme iron of cytochrome P-450 (99,100). The finding that different spectral changes can be produced by different substrates (95-98) suggests the presence of more than one binding site on cytochrome P-450, at least in the microsomal system. While cytochrome P-420 still appears to retain substrate-binding capacity, the very weak spectral change observed on substrate - P-420 interaction (90,98) suggests a greatly reduced affinity of this cytochrome for substrate. Thus the hydrophobic nature of cytochrome P-450 is important
in substrate interaction with this hemoprotein.

The present experiments suggest that the interaction of deoxycorticosterone with cytochrome P-450 results in a stabilization of the hemoprotein. Thus if the spectral changes produced by interaction of deoxycorticosterone with cytochrome P-450 reflect a conformational change in this hemoprotein, then the altered conformation is more resistant to decomposition. Of interest also is the finding that deoxycorticosterone can interact with the oxidized state of cytochrome P-450 as evidenced by the spectral changes produced in the absence of any reducing agent. Indeed it has been reported that total chemical reduction of the cytochrome eliminates the substrate-induced spectral changes (86,87,95-98). Cammer and Estabrook have observed however (85) that enzymatic reduction of cytochrome P-450 under anaerobic conditions resulting in total reduction of the hemoprotein, does not eliminate the deoxycorticosterone-induced spectral change in adrenal mitochondria. They suggest that chemical reduction with dithionite modifies deoxycorticosterone interaction with cytochrome P-450 but that this modification does not occur when the hemoprotein is reduced enzymatically via the cytochrome P-450 respiratory chain. In the present studies it is observed that NADPH does not alter the stabilizing effects of deoxycorticosterone or Metopirone on cytochrome P-450.

Both dicumarol and Metopirone exhibit pronounced effects on cytochrome P-450. The evidence that deoxycorticosterone
interacts with cytochrome P-450 and that this hemoprotein is both the oxygen-activating and substrate-activating component of 11β-hydroxylation leads to the conclusion that the effects of Metopirone and dicumarol on this hemoprotein are related to their inhibitory actions on 11β-hydroxylation.

The effect of dicumarol on cytochrome P-450 is complex; at least two distinct effects are discerned. At low concentrations dicumarol diminishes the rate of decomposition of cytochrome P-450 thus increasing the recovery of this hemoprotein. The profile of the increased recovery of cytochrome P-450 in the presence of low dicumarol concentrations is very similar to that observed with deoxycorticosterone. However, the protective influence of dicumarol is not as great as that observed with deoxycorticosterone. Spectrophotometric evidence for the direct interaction of dicumarol with cytochrome P-450 could not be obtained because of the absorption of this compound in the soret region of the spectrum. The similarity of the protective influence of dicumarol to that of deoxycorticosterone implies that the inhibitor binds to the cytochrome and increases its stability. However, the protective effects of deoxycorticosterone and dicumarol are additive. Also dicumarol, at concentrations that stabilize cytochrome P-450, is a noncompetitive inhibitor of 11β-hydroxylation. These two facts suggest that dicumarol and deoxycorticosterone are binding to separate sites on the cytochrome and that the binding of one compound does not interfere with the binding
of the other. This is supported by the finding that low concentrations of dicumarol do not affect the spectral changes produced by the binding of deoxycorticosterone to cytochrome P-450. The inhibition of 11β-hydroxylation exhibited by dicumarol suggests that this compound interferes with the interaction of the substrates of the hydroxylase system, i.e., oxygen and deoxycorticosterone, either by binding to a site on cytochrome P-450 between oxygen and the steroid or by altering the conformation of the hemoprotein in such a manner as to reduce the interaction of the two substrates and hence reduce the rate of 11β-hydroxylation. Alternatively the binding of dicumarol could be inhibiting the attachment of oxygen to the heme portion of cytochrome P-450. There is some evidence that at low concentrations dicumarol also protects cytochrome P-420 from decomposition (Fig. 45). Omura and Sato (89) have demonstrated that the destruction of cytochrome P-420 is accompanied by a loss of protoheme indicating a breakdown of the heme moiety of this cytochrome. Thus dicumarol may be binding to, or interacting directly with the heme of cytochrome P-450 increasing its stability but inhibiting its binding of oxygen. Either of the above mechanisms for dicumarol inhibition of 11β-hydroxylation would yield noncompetitive kinetics with regard to substrate deoxycorticosterone as has been demonstrated.

As the dicumarol concentration is increased, a second effect becomes apparent. At high concentrations dicumarol
increases the rate of breakdown, and hence the instability of cytochrome P-450, an effect directly opposed to that observed at low dicumarol concentrations. Therefore under these conditions dicumarol alters cytochrome P-450 in such manner that the hydrophobic environment around the heme is no longer maintained, with the resulting formation of cytochrome P-420. A similar conversion of cytochrome P-450 to cytochrome P-420 can be achieved by a wide variety of agents including deoxycholate, urea, proteases, phospholipase, sulfhydryl reagents and organic solvents (88-94). Whether the dicumarol-produced conversion of cytochrome P-450 to cytochrome P-420 proceeds by a primary effect on hydrophobic interactions of the hemoprotein in a manner similar to that of detergents or urea, or by a secondary effect through some conformational change in the protein remains to be elucidated.

The inhibition of 11β-hydroxylation produced at high concentrations of dicumarol is likely a result of the enhanced conversion of cytochrome P-450 to cytochrome P-420 caused by dicumarol at these concentrations. Cytochrome P-420 does not function in 11β-hydroxylation. The effects of high concentrations of dicumarol on both 11β-hydroxylation and cytochrome P-450 can be diminished by an increase in steroid substrate concentration. The second inhibitory action of dicumarol on 11β-hydroxylation can be almost eliminated at high concentrations of deoxycorticosterone. Cytochrome P-450 recoveries at any given dicumarol concentration in the region where the
destruction of this hemoprotein is evident are increased in the presence of deoxycorticosterone, the increase being greater than that observed in the presence of deoxycorticosterone alone. The dicumarol concentration at which decomposition of cytochrome P-450 is initiated is also higher in the presence of deoxycorticosterone. Under experimental conditions where high dicumarol concentrations do not produce conversion of cytochrome P-450 to cytochrome P-420, this compound can inhibit the spectral change observed on addition of deoxycorticosterone to the cytochrome P-450-containing enzyme fraction... This inhibition can be diminished by an increase in substrate concentration. Thus at high dicumarol concentrations there is competition between deoxycorticosterone and dicumarol for cytochrome P-450 binding. This implies that these two compounds either bind at the same or overlapping sites, or that the conformational change induced in the hemoprotein by one compound prevents the binding of the other. If dicumarol does produce such a conformation change in cytochrome P-450, then in addition to being unable to bind substrate, the hemoprotein becomes unstable and is converted to cytochrome P-420.

The effect of Metopirone on cytochrome P-450 is analogous to that produced by deoxycorticosterone and dicumarol at low concentrations. Low concentrations of Metopirone stabilize the cytochrome resulting in an increase in recovery of cytochrome P-450 after incubation in the presence of this compound. The concentration at which Metopirone stabilizes
cytochrome P-450 is in the region of the $K_i \ (1.0 \times 10^{-7} \text{ moles/l})$ for inhibition of $11\beta$-hydroxylation and indicates that the two processes are related. At this concentration Metopirone also competitively inhibits the deoxycorticosterone-induced spectral change in the cytochrome. This effect is similar to that produced by high dicumarol concentrations, except that conversion to cytochrome P-420 does not occur. The competitive kinetics of Metopirone inhibition of the $11\beta$-hydroxylation of deoxycorticosterone, the similar protective effect of these two compounds on cytochrome P-450, and the ability of Metopirone to inhibit the binding of deoxycorticosterone to cytochrome P-450 are all consistent with the hypothesis that either the two compounds bind at the same site on cytochrome P-450, or that the conformational change induced by the binding of one compound can competitively prevent the binding of the other. Although no spectral interaction between cytochrome P-450 and Metopirone at these concentrations can be observed, it is possible that the intensity of the spectral change is too low to be detected.

An increase in the Metopirone concentration produces a further alteration in cytochrome P-450. There appears to be a rapid conversion of this hemoprotein to a second stable form. This conversion is accompanied by an initial loss of cytochrome P-450 as measured by the spectral change at $450 \ \mu\text{m}$. The rapid loss of absorbance at this wavelength may indicate that higher concentrations of Metopirone alter the conformation of cytochrome P-450 in such a manner as to diminish the
extinction coefficient of the carbon monoxide-hemoprotein complex. That Metopirone at these concentrations can produce conformational changes in cytochrome P-450 is suggested by the ability of Metopirone to induce spectral changes in this hemoprotein. The Metopirone-induced difference spectrum is very similar to those observed on addition of organic solvents to microsomal cytochrome P-450 (98) at concentrations known to induce changes in protein conformation. This second effect of Metopirone on cytochrome P-450 occurs at concentrations higher than those required for inhibition of deoxycorticosterone 11β-hydroxylation and may not be responsible for its inhibitory action. The second effect may be related to the stimulation of NADPH oxidation observed at high Metopirone concentrations. The secondary effect of Metopirone can be prevented by the addition of deoxycorticosterone. Thus whatever alteration Metopirone at high concentrations produces in cytochrome P-450 cannot take place in the presence of steroid substrate.

Although the dual effects of both dicumarol and Metopirone on cytochrome P-450 have been explained in terms of two separate effects and perhaps two separate binding sites on cytochrome P-450 for each inhibitor, the possibility exists that the secondary effects of these compounds on cytochrome P-450 are merely extensions of their initial effects. Imai and Sato (90) have observed that addition of excessive amounts of substrate aniline to liver microsomes produces a conversion of cytochrome P-450 to cytochrome P-420. We have not
observed a similar conversion of mitochondrial cytochrome P-450 in the presence of deoxycorticosterone. However dicumarol and Metopirone may be acting in a manner analogous to aniline. The addition of moderate concentrations of these compounds may modify the cytochrome with a resultant increased stability. On further additions of these reagents modification of the cytochrome becomes increasingly more drastic up to a point where a new conformation is assumed. In the case of dicumarol, integrity of the cytochrome P-450 structure can no longer be maintained and this results in its conversion to cytochrome P-420. Thus the findings that both dicumarol and Metopirone appear to have more than one effect on cytochrome P-450 does not necessarily imply that there are two distinct binding sites on this hemoprotein for each inhibitor.

The mechanism of inhibition of dicumarol and Metopirone has been explained on the supposition that these compounds can induce alterations in cytochrome P-450, presumably in its conformation, that result in an inhibition of the hydroxylation process. Observations by a number of workers support the postulation that cytochrome P-450 exists in more than one conformation. The spectral changes in the hemoprotein induced by the presence of hydroxylation substrates or inhibitors have been observed in our experiments and by a number of other groups both in microsomal (87,95-98) and mitochondrial (85,86) hydroxylase systems. These changes suggest that substrates produce a conformational change in cytochrome P-450 resulting in an alteration of the environment or ligand interaction of the heme. Cammer and Estabrook (85) have studied the inter-
action of substrate deoxycorticosterone with mitochondrial cytochrome P-450 in both the oxidized and reduced states and have obtained spectral evidence for the transition of cytochrome P-450 through several states during the course of 11β-hydroxylation. Evidence for the existence of cytochrome P-450 in multiple forms has been provided by Imai and Sato (102-104) during studies on the binding of ethyl isocyanide to reduced cytochrome P-450 of rabbit liver microsomes. They found that the reduced form of this hemoprotein could bind ethyl isocyanide to yield a spectrum possessing two absorption maxima in the sorbet region at 430 μm and at 455 μm. They found that the heights of these absorption maxima were profoundly influenced by pH, in an interdependent manner. Similar, but less intense pH-dependent spectral changes were observed on binding of substrate aniline or other lipophilic agents to cytochrome P-450. They concluded that in the reduced state, cytochrome P-450 exists in two interconvertible forms that appear to be in a pH-dependent equilibrium.

While addition of substrates and inhibitors produce spectral changes in cytochrome P-450, the presence of these agents does not, in the majority of instances, alter the absorption maximum at 450 μm of the carbon monoxide complex. Cammer and Estabrook (85) have observed some deoxycorticosterone-alteration of the carbon monoxide spectrum of cytochrome P-450 in the visible region, but reported no change in the 450 μm absorption. Schenkman et al. (97) found that cer-
tain substrates of the microsomal hydroxylase produce a shift in the absorption maximum of the carbon monoxide complex, while other substrates have no effect. Therefore the various forms of cytochrome P-450 produced by addition of various agents need not differ markedly in the absorption spectra of their carbon monoxide complexes.

On the basis of experimental data presented in this thesis, it is proposed that deoxycorticosterone, Metopirone, and dicumarol can induce conformational changes in cytochrome P-450. While these changes do not alter the spectrum of the carbon monoxide complex of cytochrome P-450, they do affect the stability of this hemoprotein and its ability to function in 11β-hydroxylation. A summary of the interactions is presented in Figure 63.

Cytochrome P-450 isolated from the acetone powder of adrenal mitochondria is relatively unstable and undergoes spontaneous decomposition to cytochrome P-420. Steroid substrates such as deoxycorticosterone, and low concentrations of both dicumarol and Metopirone bind to cytochrome P-450 producing a conformational change to P-450 with increased stability. The binding of Metopirone and deoxycorticosterone to cytochrome P-450 is competitive in nature and this results in a competitive inhibition of 11β-hydroxylation by Metopirone. The competitive binding of Metopirone and deoxycorticosterone to cytochrome P-450 suggests that the two compounds may either bind to the same site or that the conformation change induced
Fig. 63. Schematic representation of the conversion of cytochrome P-450 to different conformational states by interaction with deoxycorticosterone, Metopirone, and dicumarol.
by the binding of either compound prevents the binding of the second compound. Dicumarol binding at low concentrations to cytochrome P-450 is noncompetitive with deoxycorticosterone as the stabilizing effects of these two compounds are additive. Therefore they must bind at different sites. The binding of dicumarol results in noncompetitive inhibition of 11β-hydroxylation suggesting that the binding of this inhibitor interferes with the interaction of deoxycorticosterone and oxygen, the substrates for 11β-hydroxylation.

While the scheme outlined in Figure 63 indicates that deoxycorticosterone, dicumarol, and Metopirone convert cytochrome P-450 to a more stable form, cytochrome P'-450, it is not meant to suggest that the binding of each of the compounds produces the same conformational change in the hemoprotein. It is quite likely that deoxycorticosterone, dicumarol, and Metopirone each modify cytochrome P-450 in a different manner. The term cytochrome P'-450 is used in Figure 63 merely to denote that the binding of these compounds produces a more stable form of cytochrome P-450.

High concentrations of Metopirone result in a further alteration of cytochrome P-450 to a form designated, P''-450. This form is also stable but a loss of some cytochrome P-450 occurs during conversion to this second form. It is also possible that the carbon monoxide complex of the cytochrome P''-450 has a diminished extinction coefficient when compared to that of the carbon monoxide complex of cytochrome P'-450. For-
mation of P''''-450 is prevented by the presence of deoxycorticosterone.

High concentrations of dicumarol result in the formation of P''''-450 which is extremely unstable and very rapidly converted to cytochrome P-420. The binding of dicumarol at high concentrations appears to be competitive with deoxycorticosterone; hence the formation of P''''-450 is diminished by addition of the steroid substrate. The competitive binding of dicumarol and deoxycorticosterone to cytochrome P-450 suggests that the second inhibitory effect of dicumarol on 11β-hydroxylation at high inhibitor concentrations is competitive in nature.

The phospholipid fraction of the acetone extract of adrenal mitochondria also has the ability to stabilize cytochrome P-450, as has been previously discussed. It is not known whether this is a general effect due to an increase in the hydrophobic nature of the environment of cytochrome P-450 or to a specific binding of phospholipid with a resulting conformational alteration. Evidence also indicates that the phospholipid fraction may mediate some form of reconstitution of cytochrome P-450 from cytochrome P-420 presumably due to a recombination of lipid with the hemoprotein moiety.

It is apparent that the structure of cytochrome P-450 that gives this hemoprotein its unusual properties is maintained by hydrophobic bonding within the cytochrome complex. Deoxycorticosterone, dicumarol, Metopirone, and phospholipid all appear to exert some influence on the hydrophobic nature
of this hemoprotein. The structural features of deoxycorticosterone, Metopirone, and dicumarol that allow these compounds to bind to cytochrome P-450 are as yet unclear. The substrates for both the microsomal and mitochondrial hydroxylase systems are generally lipophilic in nature. Imai and Sato (104) have stated that only lipophilic agents appear to cause spectral changes in cytochrome P-450 associated with their binding. Deoxycorticosterone, Metopirone and dicumarol all are cyclic compounds possessing lipophilic properties. Yet it is unlikely that this general property is responsible for their binding to this cytochrome. Corticosterone and adrenosterone also are lipophilic, yet they have no ability to stabilize cytochrome P-450. Therefore the interactions of deoxycorticosterone, Metopirone, and dicumarol with cytochrome P-450 exhibit some degree of specificity.

The structural features of Metopirone and dicumarol that permit these compounds to compete with deoxycorticosterone for binding to cytochrome P-450 are unclear. While these compounds are all cyclic, they are not structurally similar. It is therefore unlikely that they are binding to the same site on the hemoprotein. It must be concluded that cytochrome P-450 is complex and possesses multiple sites for the binding of such compounds. The binding of one compound must alter the conformation of the binding sites of the other compounds in such a manner as to prevent their binding to the hemoprotein.

The ultimate elucidation of the mechanism of inhibition by dicumarol and Metopirone must await an understanding of
the complex nature of cytochrome P-450. However all attempts to purify this hemoprotein have been unsuccessful due to its instability. We suggest that studies on the effects of substrates and inhibitors of the 11β-hydroxylase on cytochrome P-450 and 11β-hydroxylation may give much information as to the characteristics of the substrate active site and the inhibitor sites on cytochrome P-450, and the relationships between these sites. Such a study may ultimately lead to a greater knowledge of the overall mechanism of 11β-hydroxylation.
BIBLIOGRAPHY


100. Ichikawa, Y., Hagihara, B., and Yamano, T., Arch Biochem. Biophys., 120, 204 (1967).


158. Gotterer, G.S., Biochemistry, 6, 2147 (1967).

165. Scarano, E., Geraci, G.; and Rossi, M., Biochemistry, 6, 192 (1967).

166. Rothman, L.B., and Cabib, E., Biochemistry, 6, 2098 (1967).

167. Robinson, J.D., Biochemistry, 6, 3250 (1967).


