Studies on steroid hydroxylases--with particular emphasis on their electron transports

By Tokuji Kimura

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By Tokuji KIMURA, (Laboratory of Biochemistry, College of Science,
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Introduction

Many excellent papers have been published on a variety of studies made on the synthesis of steroid hormones in animal organs and on bacteria. The present report describes the steroid hydroxylases identified in mitochondria by centrifugation after the break down of cells in the process of synthesis of steroid hormones with cholesterol as starting material. Hydroxylases known to be falling under this category are as follows:

1) Cholesterol-20β-hydroxylase
2) Cholesterol-22-hydroxylase
3) Cholesterol desmolase
4) Steroid-11β-hydroxylase (EC 1.14.1.8)
5) Steroid-18-hydroxylase

Besides these hydroxylases, steroid-21-hydroxylase [EC 1.14.1.8], steroid-17α-hydroxylase [EC 1.14.1.7] are known to be present in microsomes. In adrenal cortex, the synthesis of corticoids from cholesterol are enhanced by 3β-OH steroid dehydrogenase [EC 1.1.1.51] and steroid-Δ-1 isomerase [EC 5.3.3.1] together with these hydroxylases. Their activities indicate inclusion of hydroxyl group into steroid molecules irrespective of kyokuzaisei (polarity)
of cell granules using NADPH as reducing agent and requiring molecular oxygen. According to the classification of oxygenases, these belong to monooxygenases and the entire process of the chemical reaction may be shown in the following formula:

\[ AH + NADPH + O_2 \rightarrow A-OH + NADP^+ + H_2O \]

Similarly to the chemical reaction of monooxygenase, in general, one atom of molecular oxygen is included in steroid particles and the other to form water. However, as discussed later in this report, the stoichiometry of this reaction is not yet established because these hydroxylases contain autoxidation system of NADPH.

The reason for the author's particular interest in hydroxylases found in mitochondria may be attributed to the following:

1) Since strong electron transmission system which conjugate with oxidative phosphorylation is found in mitochondria, how would this electron transmission system functionally effect electron transmission system of hydroxylation and what would be the microstructural relation of the former with the latter in the granule.

2) Comparison with mechanism of non-specific hydroxylation primarily in connection with detoxication in physiological terms, (for example, hydroxylation of aniline, etc.,) which are identified in microsomes of liver etc.
3) Comparison of electron transmission mechanism of steroid hydroxylases which are considered to be granular complex and that of ferroflavoproteins also considered as granule but which required a special procedure for solubilization. Among these ferroflavoproteins are, for instance, succinic dehydrogenase [EC 1.3.99.1] containing four atoms of iron (non-hemin) and a mole of FAD (flavin adenine dinucleotide), NADH dehydrogenase, choline dehydrogenase (EC 1.1.99.1) and α-glycerophosphate dehydrogenase (EC 1.1.2.1).

4) Enzymes that participate in cholesterol decomposition are found separately in mitochondria and microsomes. The source or site of formation of these enzymes within the cells and significance of their kyokuzaisei (polarity).

5) Presumable action of peptide hormones on mitochondria, direct or otherwise, may be inferred from the presence of the enzymes in mitochondria which are in such phase of metabolism as to be receiving stimulus from ACTH (adrenocorticotropic hormone).

I. Cellular Environment of Steroid Hydroxylases

It is necessary to investigate the physiological environment of steroid hydroxylases, if we were to understand them under the inner cellular physiological conditions. Thus, determination of weights of such cofactors as are pertinent to hydroxylation, NADP and NADPH in particular, was carried out.
Activities of enzymes in NADPH supplying system as well as activities of those in NADPH consuming system which are likely to be competitive with hydroxylases were also determined. Special attention was paid to physiological environment of hydroxylases on the basis of the statement made to the effect that ACTH acts to increase the concentration of NADPH by stimulating glycogen decomposition. 19)

The cortex was homogenized and centrifuged as a whole without regard to the zonal difference. The quantitative analysis of enzymes and auxiliary enzymes in nucleus, mitochondria, microsomes, and the solubles were made separately. Table-1 shows the distribution of oxidoreductases in the adrenal cortical cells. As in other animal tissues, the major portion of glucose-6-phosphate dehydrogenase [EC 1.1.1. 49], and 6-phosphoglucon dehydrogenase [EC 1.1.1. 43] were present in the solubles of adrenal cortex and their extremely strong activities as compared with those in other organs are noteworthy, for example, the activities per tissue weight of these enzymes are as intense as 900 folds of those in heart musculature and 40 folds of those in liver, while in adrenal medulla, they do not exceed \( \frac{1}{5} \) of the strength of those in the cortex. 17) Further, the major portion of isocitrate dehydrogenase which reduces NADP [EC 1.1.1. 42] is also identified in the solubles. Although the total activities of enzymes in NADPH supply system which are found predominantly
in the solubles of the cortex may be understood in connection with NADPH requirements of steroid hydroxylases, what physiological significance the fact that activities of those enzymes in NADPH supply system is virtually 100 times stronger than the total activities of hydroxylases bears is of interest to the author. Total activities of NADPH supply system is so much stronger even if we include activities of NADPH oxidases, which are known to have intense activities among NADPH reoxidases, into those of steroid hydroxylases. This is supported by the evidence on determination of NADP and NADPH contents. 18)

Table-1.

**Distribution of Oxidase-Reductases in Adrenal Cortical Cells**

<table>
<thead>
<tr>
<th>Enzyme Activity (mmol/min)</th>
<th>Mitochondria</th>
<th>Microsome</th>
<th>Specific Activity (umol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>160</td>
<td>70</td>
<td>520</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase</td>
<td>90</td>
<td>64</td>
<td>360</td>
</tr>
<tr>
<td>Succinate dehydrogenase (NADPH)</td>
<td>90</td>
<td>120</td>
<td>2590</td>
</tr>
<tr>
<td>Lipoamide dehydrogenase (reduced)</td>
<td>360</td>
<td>100</td>
<td>2590</td>
</tr>
<tr>
<td>CoQ dehydrogenase</td>
<td>185</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>NADPH-dependent succinate dehydrogenase</td>
<td>990</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NADPH-dependent cytochrome c reductase</td>
<td>990</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>NADPH dehydrogenase</td>
<td>846</td>
<td>717</td>
<td>2590</td>
</tr>
<tr>
<td>NADPH-dehydrogenase***</td>
<td>38</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>NADH dehydrogenase</td>
<td>6230</td>
<td>7490</td>
<td>7500</td>
</tr>
<tr>
<td>NADH dehydrogenase***</td>
<td>176</td>
<td>111</td>
<td>38</td>
</tr>
<tr>
<td>Steroid-21-dehydrogenase</td>
<td>4</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Steroid-11β-dehydrogenase</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol oxidase***</td>
<td>7×10^-1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* * * * * *

- * indicates measurement of electron acceptor.
- ** indicates measurement of electron donor.
- *** indicates measurement of electron acceptor.
- **** indicates oxidation of 1 g of adrenal cortex to measure activity of NADPH dehydrogenase.

(Mori, M., Nakamura, T., 1974.)
### Table-1. Distribution of Oxidoreductases in Adrenal Cortical Cells

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Mitochondria</th>
<th>Microsomes</th>
<th>Solubles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>160</td>
<td>70</td>
<td>3520</td>
</tr>
<tr>
<td>6-phosphoglucon dehydrogenase</td>
<td>90</td>
<td>64</td>
<td>3040</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase (NADH)</td>
<td>90</td>
<td>120</td>
<td>2390</td>
</tr>
<tr>
<td>Malic acid dehydrogenase (decarboxylate)</td>
<td>360</td>
<td>100</td>
<td>1270</td>
</tr>
<tr>
<td>6-phosphoglucon dehydrogenase</td>
<td>90</td>
<td>120</td>
<td>2390</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase (NADH)</td>
<td>90</td>
<td>120</td>
<td>2390</td>
</tr>
<tr>
<td>Malic acid dehydrogenase (decarboxylate)</td>
<td>360</td>
<td>100</td>
<td>1270</td>
</tr>
<tr>
<td>Succinic dehydrogenase* (phosphatase)</td>
<td>185</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>α-glycerophosphate dehydrogenase*</td>
<td>990</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Choline dehydrogenase*</td>
<td>0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>NADPH dehydrogenase**</td>
<td>846</td>
<td>717</td>
<td>5630</td>
</tr>
<tr>
<td>NADPH oxidase***</td>
<td>35</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
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<td>6230</td>
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<td>7500</td>
</tr>
<tr>
<td>NADH oxidase***</td>
<td>176</td>
<td>111</td>
<td>38</td>
</tr>
<tr>
<td>Steroid-21-hydroxylase</td>
<td>4</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Steroid-11α-hydroxylase</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol side chain breaking enzyme**</td>
<td>7x10⁻²</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* take phenazine methosulfate as electron receptors.
** take ferricyanide as electron receptors.
*** take molecular oxygen as electron receptors.
****/Beef adrenal cortex; the figures indicate activity converted into unit of μmol/min per one g of poke adrenal cortex. (Kimura, Nakamura; unpublished data.)
Table-2  Distribution of NADP and NADPH\textsubscript{2} in the Adrenal Cells\textsuperscript{18}

<table>
<thead>
<tr>
<th>Cell Fractions</th>
<th>NADP</th>
<th>NADPH</th>
<th>NADPH/NADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>3.0</td>
<td>3.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Microsomes</td>
<td>&lt; 1</td>
<td>1.5</td>
<td>&gt; 1.5</td>
</tr>
<tr>
<td>Solubles</td>
<td>10.9</td>
<td>55.2</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Figures indicate μmol of pyridine nucleotides/g of the adrenal cortex.

Table-2 above shows distribution of NADP and NADPH\textsubscript{2} in the adrenal cortex cells. A major portion of NADP and NADPH are found present in the solubles and the ratio of NADPH/NADP amounts to approximately 5, while the same ratio in the mitochondria and the microsomes are respectively about one. From this, it may be learned that there are more reductases in the solubles and, quite reversely, that the presence of oxidases is also significant in the granules. Consequently it may be assumed that NADPH must be supplied from the solubles to hydroxylases in the granules. A tentative calculation of enzyme activities in the cell shows the inner cellular concentration of NADP + NADPH of glucose-6-phosphate dehydrogenase to be approximately 0.1 mM, which is close to $K_m(2 \times 10^{-5} \text{M})$ against NADP. The inner cellular concentration of glucose-6-phosphate of dehydrogenase is about 0.1 mM, which is also close to $K_m (6 \times 10^{-5} \text{M})$ against it. This means activities of about \( \frac{1}{3} \) of \( V_{\text{max}} \) are maintained under the steady state of the cells.
In sharp contrast to this is $K_m$ of granular hydroxylases against NADPH which indicates a high value of $1 \times 10^{-3}$ M due to the low membrane permeability. This means about $\frac{1}{100}$ of $V_{max}$ under the steady state in the cell, revealing anomalous imbalance also in the physiological conditions of NADPH supply system and hydroxylases. Judging from the absence or scantiness of re-oxidaases with the comparable activity of NADPH supply system in the adrenal cortex, it may be inferred that this scantiness of NADP serves to limit the rate of the total systems. Figure-1 describes schematically the speed regulatory stages as seen between the granule membrane permeability of NADPH and its reoxidaases in the adrenal cortex.

**Figure-1. Polarity and Membrane Permeability of NADP and NADPH in the Adrenal Cortex Cells**

"Cytoplasm"

"** Membrane"

"**** Granules"

On the other hand, although there are enough cholesterol present in the adrenal cortex as starting material for the synthesis of corticoids, phospholipids and those in micelle seem to
be actually utilized as substrates. Molecular form of cholest erol insoluble to water as substrate is not yet known.

II. Properties of the Adrenal Cortical Mitochondria

The mitochondrion of the adrenal cortex is not much different from that of heart musculature. Morphologically, it gives the electron micrograph as shown in Figure-2 below.

Figure-2. Electron Micrograph of Adrenal Cortical Mitochondrion

* Magnification: 30,000 ** HIDA OHI Electron microscope (HU-11B) nagetive
*** PTA ∩ Dye method (Kimura, Nakamura; unpublished)

Its diameter being 1 - 2μ, considerably well developed crista tubular ampullaris feature its property. And moreover the sizes of
the slices are at variance, which are said to have a relatively strong pleomorphic tendency. In the mitochondrion sample considered to be almost homogeneous after the centrifugation, succinic acid oxidases and NADH oxidases are present and the synthesis of ATP (adenosine triphosphate) by oxidative phosphorylation and the respiratory control function by ADP/ATP are observed. However, activities of succinic acid dehydrogenases are weak and the binding FAD contents from which the amount of the said dehydrogenases may be assumed directly is as low as about 1/7 of the heart musculature mitochondria and about 1/3 of the liver mitochondria, while activities of α-glycerol-3-dehydrogenases are somewhat stronger, amounting to approximately 1/3 of the brain mitochondria.

Chemical assay of the mitochondrion shows that there are approximately 1.7 μmol of the total pyridine nucleotides per mg of protein, about 0.6 μmol of the total flavin per mg of protein, and about 1.9 μmol of iron (non-hemin) per mg of protein. From the total iron (non-hemin), it is possible to separate non-hemin iron protein (Adrenodoxin) with 40% yield. On the constituents of cytochromes, Spiro and Ball already reported that the presence of B, C, G, A, and A3 are identified and that the contents of cytochrome c in the mitochondria of the cortex is about 1/7 of that in the heart musculature. In the above analytical data, it is difficult to find any peculiarity from those of other mitochondria. However, activities of the electron transmission system
in the cortex are by far the weaker than those in the heart musculature mitochondria. This may be attributed to the smaller requirement for the biosynthesis of ATP in the adrenal cortical mitochondria for their functional fulfillment than the requirement in the heart musculature. The adrenal cortical mitochondria is found to contain hemoproteins which are peculiar to the mitochondria. And as would be stated later, they contain cytochrome b, which is CO-binding pigment. This pigment is originally found in microsomes, which shows the maximum absorption at 450 μm in formation of the reduced CO bond whereas after the solubilization by cobra venom treatment, its maximum absorption appears at 420 μm in the formation of CO bond, and a, b absorption bands are weak. In the sample of the mitochondria, about 0.4 μmol of this CO-binding pigment is found present per mg of protein, which corresponds roughly to \( \frac{1}{5} \) of that contained in the liver microsomes. Further, flavoproteins somewhat similar to NADPH dehydrogenases which are dominant in the microsomes are also identified in the sample. It might be added that this sample appeared to be almost homogeneous on preparation. On the basis of the above factual observation, the possible mixture of microsomes in the sample of the adrenal cortical mitochondria which are considered to be almost homogeneous under the electron microscope may be suspected. In the same sample, distribution of succinic dehydrogenase and that of steroid-12α-hydroxylases agreed,
while distribution of RNA( ) and that of steroid-11\(\beta\)-hydroxylases did not.

On the chemical components of the cytochromes of the adrenal medulla, there have been reports published by Spiro, Ball,\(^{63}\) and Krisch.\(^{64}\) The presence of cytochrome b peculiar to adrenomedullary microsomes was found by Ichikawa and Yamano.\(^{65}\)

Next, on the interaction of the electron transfer system of hydroxylases and the electron transfer system of oxidative phosphorylation, it had already been pointed out by Hayashi\(^{22}\) that affinity of hydroxylases and that of cytochrome a to molecular oxygen differed from each other, the latter being approximately 10--100 times higher, while there was practically no difference in \(K_m\) of the former and the latter against reduced pyridine nucleotide. This leads to a possibility in which oxygen might be used up first by cytochromes rather than by hydroxylases. In fact, it has been noted in the adrenal mitochondria, while active phosphorylation, almost all the oxygen was consumed by cytochromes and nearly no hydroxylation occurred simultaneously. For the same reason, cytochrome c may act as a strong inhibitor on hydroxylation. This, however, may be prevented by supplying plenty of oxygen on open systems without restriction. Consequently, it was assumed that hydroxylases and phosphorylases are competitive at the molecular oxygen level. Furthermore, in the mitochondrial sub-granular fraction containing only hydroxylases, where the autooxidation system from NADPH to \(O_2\) exists, only 3% of the total \(O_2\) consumption of NADPH\(^1\) was found to be utilized for hydroxylation. In this case, too, addition
of cytchrome c has markedly increased the absorption rate of 
O₂ and at the same time inhibited hydroxylation of steroid. 
As was stated earlier, the presence of strong NADPH generating 
system in the adrenal is corroborated, although its efficiency in actual 
utilization for hydroxylation is extremely low because of the major loss incurred in the autoxidation 
system which has no explicit physiological significance.

It might be possible, theoretically, to assume the utilization of NADH for hydroxylation instead of NADPH, however, due to the excessive requirement of NADH for this purpose, the required high concentration of NADH would be physiologically unthinkable. Thus, because of the low enzyme activity for hydroxyl transport from NADH to NADPH, it is not considered probable that the utilization of NADH in the living body would actually take place.

Cholesterol Side-Chain Cleavage System¹⁻⁷)

The first step in biosynthesis of steroid hormone is the cleavage of a bond between Carbon atoms²₀ and ²₂. This reaction is caused first by hydroxylation at C₂₀ and then C₂₂, followed by the formation of dihydroxy and by consumption of another molecule of oxygen presumably to form C-O-O bond, leading to the biosynthesis of pregnenolone and isocapric aldehyde which is subsequently oxidized to isocapric acid. This metabolism has been established independently by Shimizu, Dorfman et al²,³,⁵,⁶) and Tchen et al¹,⁴) Although it has been assumed that at least three different enzyme
systems are involved in the cholesterol side-chain cleavage, their presence has never been isolated from each other due to some difficulties in synthesizing of respective substrates, and to complex procedures required for determination of product contents, and to lowness in activities and stability of enzymes concerned.

For determination of enzyme activity, the easiest method would be that by which \(^{14}C\)-isocapriolic acid content is measured as product from conversion of cholesterol-\(^{2}H\) incorporating \(^{14}C\) as substrate. The presence of the cholesterol side-chain cleavage system identified in the endocrine organs including adrenals, is found most predominantly in the mitochondrial fractions in all cases.

Crude enzyme was extracted from the acetone powder of rat adrenal mitochondria with 0.1 M phosphate buffer (pH 7.4) and then fractionated with ammonium sulfate. The fractions at 0 - 50% saturation (Fraction A) and at 50-75% saturation (Fraction B) were collected. It was observed that the side-chain cleavage activity took place only when these two fractions worked together. Fraction A contained CO-binding pigment which showed the peak at 420 nm by NADPH in the presence of CO by the differential spectrum. Its reduced pyridine hemochromogen indicated the maximum absorptions at 553, 524, and 414 nm and was considered to be ferroporphobilinogen. In this fraction, NADPH oxidase is contained, which is inhibited by 0.5 \(\times 10^{-2}\) M of amitarr and is able to reduce potassium ferriyanide.
dichlorophenolindophenol, and cytochrome c besides O_2. In frac-
tion B, the component considered to be similar to non-hem iron protein which will be discussed more in detail in the subsequent section of this report was identified on the basis of the differential spectrum in which the peaks were shown at 415 m\(\mu\) and 450 m\(\mu\) by \(\text{Na}_2\text{S}_2\text{O}_4\). The activity of cholesterol side-chain cleavage enzyme was found to be inhibited by approximately 70% with 0.5 mM of cytochrome c.

Considering the above properties of this enzyme system, the presence of the electron transfer system derived from NADPH and identical to that of steroid-13\(\beta\)-hydroxylases has been deduced.

Steroid-13\(\beta\)-Hydroxylase\(^{8,9,25,26,27}\)

These are found present exclusively in adrenals and are the complex consisting of at least more than three proteins. It is generally accepted by many scientists that steroid-13\(\beta\)-hydroxylation is highly concentrated in the mitochondrial fraction of adrenal when it has been collected by centrifugation after the cells have been broken down in 0.25 M of sucrose solution. Partial extraction of the enzyme activity is possible with the aceton powder of adrenal mitochondria, and frozen dry powder and by supersonic treatment but overall extraction is difficult to accomplish. Since one hour centrifugation of 100,000 g of the extracts caused to precipitate a considerable portion of activities, the enzyme
may be of granular properties. The extracts may be fractionated into fraction A precipitating at 0-40% ammonium sulfate saturation and fraction B precipitating at 60-80%. Neither fraction in itself has activity and only when both are mixed can it be observed. Fraction A contains CO-binding pigment as well as NADPH dehydrogenase, whereas fraction B contains non-hemiron proteins.

Although it had been generally believed that NADPH would not pass through mitochondrial membrane, the experiment carried out under the condition of broken membrane yielded a decline in $K_m$ which indicated $2 \times 10^{-5} M$ against NADPH, almost $\frac{1}{50}$ of what would be expected in the fresh mitochondria.

**Steroid-18-Hydroxylases**

Féron$^{10}$ and Birmingham et al.$^{11}$ reported that these hydroxylases had been found present only in rat adrenals. Nakamura et al.$^{62}$ further, observed that these hydroxylases were found densely concentrated in the mitochondrial fraction. The electron transfer system of these hydroxylases may be considered to be similar to that of other hydroxylases existing in other mitochondria.

III. **Components of Hydroxylases (Steroid) in Mitochondrial Fraction**

In several steroid hydroxylases in the mitochondrial fraction, there appear to be considerable things
in common between their electron transfer systems of NADPH. Thus it is thought that only the reaction of activated hydroxylases at the last stage of hydroxylation to incorporate themselves into steroid molecules can peculiarity of respective hydroxylases be recognized. In the present report, I wish to describe here the results of the analysis of components of steroid-11p-hydroxylases on which the most detailed investigation has been carried out.

**NADPH-dehydrogenase**

The presence of NADH-dehydrogenase(NADH-cytochrome 6 reductases) in mitochondria having some influence on respiratory function, and of NADPH-cytochrome b_5 reductases, [EC1.6.2.2], NADPH-menadione reductases, and NADH dehydrogenases in microsomes, and of DT-diaphorases which act on both NADH and NADPH in the soluble fraction have been already reported. All these enzymes are flavoproteins, although due to some confusions in giving different names to the apparently same enzyme, their variety is extremely complicated. The enzyme (NADPH-dehydrogenase), for example, which accepts, first among other things, the electrons from NADPH of hydroxylases also is flavoprotein and is considered to be one of the known NADPH-dehydrogenases.

NADPH-dehydrogenases of hydroxylation system have not yet been prepared successfully in homogeneous state, but its partial purification is feasible. It has been held almost definitely that they are flavoproteins, judging from other
analogous pyridine nucleotide dehydrogenases. Although they could be extracted from the acetone powder of adrenal mitochondria with 0.15 M KCl, the better results were obtained by *Naja-Naja* Cobra venom extraction. After precipitating 100,000 g of supernatant with ammonium sulfate for 30 minutes, DEAE cellulose column was used for purification. The purest sample obtainable showed fading peculiar to flavin by reduction with NADPH or Na$_2$S$_2$O$_4$. While potassium ferricyanide and menadione were found to be good electron acceptors, cytochrome c and dichlorophenol indophenol (DCPIP) showed no such function. (TABLE-3) Inasmuch as their natural electron acceptors are non-heme iron proteins (adrenodoxin), higher than potassium ferricyanide, activity was demonstrated by adrenodoxin. These flavor enzymes are probably of simple nature without containing any metal element such as iron.

Table-3. Specificity of NADPH-Dehydrogenases to Electron Acceptors

<table>
<thead>
<tr>
<th>Electron Acceptors</th>
<th>Vmax (m mol/min/ml enzyme)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferricyanide</td>
<td>472</td>
</tr>
<tr>
<td>Menadione</td>
<td>250</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>45</td>
</tr>
<tr>
<td>DCPIP*</td>
<td>35</td>
</tr>
<tr>
<td>Adrenodoxin</td>
<td>606**</td>
</tr>
</tbody>
</table>

*DCPIP: dichlorophenol indophenol

** Figure does not represent Vmax. The value was calculated from decrease of absorbancy at 414 μm with Emm =3.2/Fe.

(Kimura: Unpublished Data)
Non-Heme-Iron Protein (Adrenodoxin)$^{26,27}$

Although the presence of a component which indicate specific absorptions in purification stage of steroid-11β-hydroxylases had already been suggested by Tomkins$^8$ as early as in 1958, no determination has since been made on whether or not the component participate in hydroxylase activity and it has been left without detailed investigation for determination of its properties. Quite by accident, the author observed, during purification operation of the cholesterol side-chain cleavage system as well as steroid-14α-hydroxylases, the presence of reddish brown components which resemble each other and, moreover, which actively participated in hydroxylation in both cases. The further and thorough purification of this component produced protein containing low molecular iron. The method employed for purification is by extraction of enzyme from the mitochondrial acetone powder with 0.15 M KCl, followed by fractionation with ammonium sulfate. By repeating DEAE cellulose column process twice and through Sephadex treatment, the pure sample may be obtained ultracentrifugally. Still easier procedure would be to treat the frozen and preserved total homogenate by supersonic process and then fractionate it with ammonium sulfate, following which by the same procedure as the above mentioned method, the pure sample may be obtained. With technical deviations taken into consideration, either method would yield (equally almost) identical samples per adrenal tissue weight. That this component is indispensable for 11β-
hydroxylation is clear from the titration curve (Figure-3) obtained with use of a given amount of the further purified sample of fraction A (ammonium sulfate saturation at 0-40%) and by addition of the different amounts of this component to it.

**Figure-3. Requirement of Non-Heme-Iron Proteins (Adrenodoxin) for 11β-Hydroxylation**

* Corticosterone (µg)

** Ironprotein (mg)

In the presence of given amount of fraction A, adrenodoxin was added in different quantities. Then, the amounts of corticoids generated at each addition of adrenodoxin were determined according to Mattingly method. 

Table-4 shows the comprehensive data on physical and chemical properties of the pure sample obtained by ultracentrifugation. (Table-4)
Table-4. Properties of Adrenodoxin

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe (μ atom/mg protein)</td>
<td>103</td>
</tr>
<tr>
<td>Unstable S</td>
<td>100</td>
</tr>
<tr>
<td>S/Fe</td>
<td>0.97</td>
</tr>
<tr>
<td>$S_{20, W}(S)$</td>
<td>1.7</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>22,000 -- 23,000</td>
</tr>
<tr>
<td>$E'_{o}$ pH 7.4 (Volt)</td>
<td>0.15</td>
</tr>
<tr>
<td>Flavin</td>
<td>Not identified</td>
</tr>
<tr>
<td>Heme</td>
<td></td>
</tr>
<tr>
<td>O.D. 414/O.D.280</td>
<td>0.76</td>
</tr>
<tr>
<td>$E_{mM}(cm^{-1})$ at 414 μ</td>
<td>4.9</td>
</tr>
<tr>
<td>$A_{EMM}(cm^{-1})$ at 414 μ</td>
<td>4.2</td>
</tr>
<tr>
<td>$E_{mM}(cm^{-1})$ at 455 μ</td>
<td>4.4</td>
</tr>
<tr>
<td>$A_{EMM}(cm^{-1})$ at 455 μ</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* Difference between iron protein and protein without iron. (Suzuki, Kimura: Unpublished Data)

The component obviously contains neither flavin nor heme, nor such metals as Mn, Ni, Co, Cu, Zn, and Mo except iron as indicated in the results of analysis by atom absorption process. Total iron contents in the component determined by incineration corresponds with that of non-heme iron by chloroacetic acid extraction. Therefore, the component is considered to be non-heme iron protein. However, in view of the possible presence of other non-heme iron proteins in the adrenal mitochondria, the author wishes to propose the trivial name, "adrenodoxin" for the component until such time as the systematic designation for it may become fairly established.
Its molecular weight is about 22,000 and in one molecule, there is one to two iron atoms. Iron atoms in the protein are relatively easily dissociated during preparation or in preservation. The protein which had lost iron was observed to retain the same molecular weight of the iron containing protein, however, isolation of which was found difficult by the ordinary method. In the natural protein are two iron atoms in one molecule and the presence of those apparently with one iron atom may be interpreted as a result of mingling of protein without iron.

It has been observed that the protein, when acidified or boiled readily fades its colour. It also gives H₂S odour. This may suggest the presence of so-called "labile S". Estimation of H₂S by methylene blue process indicated the presence of 0.9 mol of H₂S per one iron atom. This demonstrates that the ratio of iron against "labile S" to be 1:1, which is similar to those of other non-heme iron proteins containing "labile S". The colouring substance derived from adrenodoxin exhibited the identical absorption spectrum with that of known methylene blue derived from Na₂S, suggesting that which is derived from the protein indeed is H₂S itself. (Figure-4)

Figure-4. Methylene Blue Absorption Spectrum Derived from Unstable Sulfur of Non-Heme Iron Protein (Adrenodoxin)
*** After acidifying adrenodoxin, collected the generated gas and made it colour as methylen blue according to Fogo et al.\textsuperscript{67}

The absorption spectrum of adrenodoxin is illustrated in Figure-5, in which the visible absorptions are indicated at 414 m\(\mu\), and 455 m\(\mu\), and a small absorption at 330 m\(\mu\), and still smaller one for protein at 280 m\(\mu\). During retention, the absorption enlarged in the region of 280 m\(\mu\) with gradual diminishing of the visible absorptions until they totally disappeared. Significant amount of iron was liberated from protein in freezing and drying of adrenodoxin. Therefore, its steric structure is considered somewhat unstable. In fact, it was found inactivated when \(\alpha\)-helix was broken, owning to resultant loss of iron atoms and "labile S". On the other hand, it can stand 10 days preservation at -20\(^\circ\)C although
\(0^\circ C\) preservation is more desirable. \(\text{EmM}\) per one iron atom of adrenodoxin at 414 m\(\mu\) being 4.9, it coincides with \(\text{EmM}\) per iron at the absorption maximum wavelength of such other non-heme iron protein as ferredoxin \(^{38,39}\) and of PPNR\(^{39,42}\) and of ferroflavin enzymes\(^{49}\) except for flavin, all of which is also about five. (Figure-5. See p25)

Elucidation on bond formula of iron atom and protein must await future investigations, however, it may be predicted from what was exhibited in the visible range of the absorption spectrum and its strength (log \(\varepsilon = 3\) ) that the bond must be of the electron charge transport type with \(S, N\) and \(O\) coordinatin in iron atom and protein. Titration of the visible absorption by PCMB show that with exactly 4 molecules of PCMB per one iron atom, fading and saturation occurred. At the same time, the absorption of mercaptide at 250 m\(\mu\)^{43} which was brought about by binding of PCMB and cystein reached saturation. Thus, it is possible to complete liberation of one iron atom with 4 molecules of PCMB. Generally speaking, reaction between PCMB and protein is expected to cause some variations in bond weight depending on time and temperature involved. In the case of the said protein, however, since PCMB reacted quite readily without any sign of being influenced by temperature factor, it is likely that iron atom is exposed on the surface. Furthermore, one molecule of \(H_2S\) binds with two molecules of PCMB with resultant formation of a substance exhibiting an absorption maximum at 250 m\(\mu\). Of the total four molecules
Figure-5. Absorption Spectrum of Non-Heme Protein

(Adrenodoxin)

* Absorbancy

** Wavelength
of PCMB, two bind with H\(_2\)S and the remaining two with cysteine in the protein. This is supported by the evidence that in the protein freed from H\(_2\)S by hydrochloride acid, binding of only 2 molecules of PCMB occurred. Use of Hg\(^{2+}\) and Cu\(^{2+}\) yielded similar result, in which case, Hg\(^{2+}\) and Cu\(^{2+}\) bind with protein in exchange of iron atom. On the basis of the above findings, it has been figured out that difference of the visible absorptions at 414 m\(\mu\) between the proteins with and without iron was 4.2 mmM per one atom of iron. Assuming iron atom coordination to be regular octahedron with six coordinations, it must consist of cysteine 2, H\(_2\)S 1, and probably histidine 2, and H\(_2\)O 1, although the exact coordination is subject to further examination except for the first two. As to how H\(_2\)S coordination actually takes place and, moreover, whether H\(_2\)S coordinates as it is or as persulfide(R-S-S-S) is still undetermined. Since both non-heme iron protein with "labile S" including the protein and the synthesized persulfide have small absorptions in the neighbourhood of 340 m\(\mu\), it is most likely that persulfide bond exists in the protein. (Figure-6)

Figure-6. Absorption Spectrum of Synthesized Persulfide
*** Persulfide was heat-synthesized in alkali with Na₂S and cystine.
When reduced by Na₂S₂O₄, adrenodoxin shows decolourizing which is quite similar to that reduced by PCMB. However, a question arises here as to the fate of iron atoms whether they became reduced or liberated by denaturation of the protein. As will be stated later, it appears that at least a part of the reduced iron retain the bond with the protein, on the basis of the facts that the dissociated iron from the protein exists in trivalence state and that the original spectrum is regained as partial oxidation takes place by enzymatic activity in the air after Na₂S₂O₄ reduction. Since it is generally accepted that bivalent iron complex salt does not display intense absorptions in the visible range, the visible absorptions of the reduced protein may as well be weak. Reduction is not feasible for ascorbic acid or BH₄ inasmuch as no change is to be expected in the visible absorptions. Notwithstanding the complex problems involved in the chemical reduction of the protein such as denaturation, and as a result of reduction of the protein under anaerobic condition with NADPH dehydrogenase and NADPH as reducing agents, marked bleaching was observed at given intervals of time in the visible absorptions. The spectrum at the time of bleaching was similar to those by DCOMB and Na₂S₂O₄.

(Figure-7)

Figure-7. Enzymatic Reduction of Non-Heme Iron Protein (Adrenodoxin)
*** NADPH and NADPH dehydrogenase were added to adrenodoxin under anaerobic condition, and its bleaching was automatically recorded on the spectrum at given intervals of time.

The extent of bleaching was proportionate to the amount of reducing enzymes and was caused by NADPH and NADH. It must be mentioned here that the rate of reaction by NADH was only \(\frac{1}{100}\) of that by NADPH, as shown in Figure-8, indicating the specificity of this dehydrogenase for NADPH. However, the addition of large excess of NADH bleached adrenodoxin to the same extent as did NADPH. As aeration of the enzymatically reduced adrenodoxin slowly reoxidized it, the original spectrum with oxidized absorptions was completely regained. (Figure-8)
Figure-8. Reoxidation of Reduced Non-Heme Iron Protein (Adrenodoxin)

* Absorbancy
** Air
*** Time

**** Changes of absorbancy at 414 m\(\mu\) were measured at given time intervals. At the indication of arrows, air was introduced at Curve A and the excessive NADH was added to Curve B.

In effect, the difference in \(\Delta E_{mM}\) at 414 m\(\mu\) of the oxidized and the reduced is 3.2 per iron atom, making up of about 80% of \(\Delta E_{mM}\), the difference between the proteins with and without iron. The slow rate of reoxidation observed in the reduced may be considered as direct reaction to molecular oxygen. However, it is not yet clear whether the reaction may be understood as oxygen activation stage of steroid.
hydroxylation or as simple autoxidation. When the complete anaerobic condition is provided for the oxidized under the reduced pressure and by glucose oxidase and glucose, no change is observed in the absorption spectrum irrespective of the presence of oxygen. While molecular oxygen does not coordinate with iron atoms, perferryl ion (FeO2^2+) is formed in the reduced from bivalent iron and oxygen. On returning to trivalent state, it possibly releases activated oxygen. In view of considerably slow reaction rate in steroid hydroxylation and of its poor affinity with oxygen, this reoxidation process might serve as activation of oxygen.

Decolouration of adrenodoxin by enzymatic reduction was titrated with NADPH which was added at 414 μM. Titration results show that saturation was reached by 0.5 moles of NADPH per one atom of iron in the protein. (Figure-9)

Figure-9. Enzymatic Titration of Iron Atom in Non-Heme Iron Protein (Adrenodoxin) by NADPH^27

* Decrease in Absorbancy at 414 μM

** Molar Ratio
On reduction of adrenodoxin with a given quantity of NADPH dehydrogenase and with various amounts of NADPH decolouration was measured at 414 mλ.

Since NADPH transmits two electrons, the fact that molar ratio of NADPH/Fe was 0.5 means iron in the protein was totally reduced by NADPH and proves that all iron in adrenodoxin was functional with respect to reduction, and that conversely, no iron without function was contained.

From the already discussed coordinations of iron atom and the protein, it may be expected to have optical activity. Its flash dispersion exhibited multiple Cotton effects with large plus flash in the visible range as shown in Figure-12.

Figure-10. Flash Dispersion of Non-Heme Iron Protein (Adrenodoxin)

* Wavelength
** Oxidized
*** Enzymatic Reduction
**** Reoxidized by air

***** Enzymatic reduction was achieved under the conditions similar to those described in Figure-7. Arrows indicate the locations of absorption maxima. (α)20 was converted per concentration (M) iron atom in adrenodoxin.

(Kimura: Unpublished)
The largest flashes are those corresponding to absorptions at 414 \( \text{m}\mu \) and 455 \( \text{m}\mu \), and the wavelength of the absorption maximum at 455 \( \text{m}\mu \) and that of polarization value 0 are almost in agreement. From the above observations, the absorptions are regarded as being dependent upon one electron transport. Cotton effects disappeared on liberation of iron atom, which largely decreased on reduction by NADPH + NADPH dehydrogenase, indicating concomitant changes in iron coordination and loss in optical activity. However, on reoxidation by aeration, the original multiple Cotton effects appeared again. The fact that flash dispersions of non-heme iron protein changed with oxidation-reduction means coordinations of iron largely transformed in the oxidized as well as in the reduced, thus, leading us to believe the close relationship between the optical activity and the electron transfer system of adrenodoxin. Further, striking similarity between the flash dispersions of the oxidized adrenodoxin and PPNR\(^{45} \) may suggest its high analogy with the iron coordination structure.

In order to know the valency state of iron in adrenodoxin, although there still exist many problems from the viewpoint of chemical analysis, the extract of non-heme iron with trichloroacetic acid was made from the sample pretreated with two atoms of \( \text{Hg}^{2+} \) per one molecule of the protein to inhibit reducing function of SH radical. Reddish-brown colour of \( \text{Fe}^{2+}-\text{o-phenanthroline} \) developed only when ascorbic acid was added as reducing agent. Without \( \text{Hg}^{2+} \), reduction occurred by SH radical contained in the protein. The substance with
reddish-brown colour was found even when ascorbic acid was not added. From the result, the majority of iron (80%) was considered as Fe$^3$ in the oxidized protein. Virtually all the iron in the protein should be considered as Fe$^3$, particularly in conjunction with the afore mentioned enzymatically reduced case. (Table-5)

Table-5. Valancy State of Iron Atom in Adrenodoxin

<table>
<thead>
<tr>
<th>Additives before TCA extraction</th>
<th>Reductant for Colouring</th>
<th>Fe$^{2+}$ / Atom/ mg Protein</th>
<th>(%(Fe$^{2+}$ / TotalFe)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>Ascorbic acid</td>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td>none</td>
<td>none</td>
<td>40</td>
<td>83</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>Ascorbic acid</td>
<td>42</td>
<td>88</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>none</td>
<td>10</td>
<td>21</td>
</tr>
</tbody>
</table>

Estimation of iron was carried out by O-phenanthroline method. (Kimura: Unpublished)

Study of the electron of adrenodoxin by Electron Spin Resonance$^{35}$ revealed the narrow signals given in the regions of $g_1 = 1.94$ and $g_{11} = 2.01$ in the reduced protein. Ever since Beinert$^{45}$ reported in 1960 on $g_1 = 1.94$ signal which he found in the electron transfer system of mitochondria, its chemical constitution as source of the signal have been much discussed. For example, there are some who assume it to be bivalent iron in high spin state on one hand, and or to be a pair of iron atoms consisting of the two on the other, while there is still
other scientists who believe it to be sulfur radical rather than iron. Whatever it may be, the signal is not yet to be recognized in the known bivalent iron compounds. Among non-heme iron proteins known to date to have \( g_1 = 1.94 \) signal are succinic dehydrogenase originated in mitochondria, NADH dehydrogenase and ETP containing the same, mitochondrial aldehyde oxidase, xantine oxidase, and dioxylotine acid dehydrogenase all with "labile S". However, this signal is not found in ferredoxin (PPNR) which contains both non-heme and "labile S" and resembles adrenodoxin. In ferritine, conalbumin and hemeruthline, all of which are without "labile S" but are non-heme iron proteins, one cannot find the same signal. Although \( g_1 = 1.94 \) signal is usually found only in iron flavin enzymes, it is possible also to get \( g_1 = 1.94 \) signal in the artificial non-heme iron protein obtained by liberating flavin from iron flavin enzymes. Of pure iron proteins with explicit functions, adrenodoxin is the first to give the signal in the region of \( g_1 = 1.94 \). Another non-heme iron protein isolated from *Chromatium Azotobacter* which has high oxidation-reduction potential is known to have the same signal. However, its physiological function is not determined. The artificial non-heme iron protein in succinylated form from reduced CoQ-cytochrome \( \zeta \) reductase complex, having lost its function, is known to give the signal in the region of \( g=1.90 \) thus proving its denaturation.
Ferredoxin,\textsuperscript{39) crystallized from \textit{Clostridium pasteurianum} etc., photosynthetic pyridine nucleotide reductase from chloroplasts of plants or ferredoxin of chloroplasts (PPNR)\textsuperscript{39-42)} have striking similarity with adrenodoxin in chemical properties such as molecular weights and absorption spectra, however, they differ greatly in their oxidation-reduction potentials. Ferredoxin and PPNR have the value of -420 mV while that of adrenodoxin is +150 mV. For this reason PPNR is considered to have no steroid hydroxylation in contrast to adrenodoxin which does not have photosynthetic pyridine nucleotide reduction. Isolation of a protein identical to the adrenal non-heme iron protein has been reported two weeks after our report on the same work had been published for the first time, by Omura, Eatabrook et al\textsuperscript{46)} of Johnson Foundation, U.S.A. at the symposium held in Yellow Spring and on that occasion its relationship with steroid hydroxylation was indicated.

\textbf{CO-binding Hemoproteins:}

In 1958, Klingenberg\textsuperscript{50)} and Garfinkel\textsuperscript{51)} reported the presence in mammalian liver microsomes of CO-binding and atypical homoprotein. This was further developed by Omura and Sato\textsuperscript{52,53)} as a result of their experiments, the evidence was presented that the protein contained protoporphyrin of cytochrome of the \textit{b} type and in microsomes CO-binding (reduced) exhibited the absorption maximum of 450 m\textmu and the solubilized CO-binding at 421 m\textmu. Moreover, its close relationship with microsomal aniline hydroxylation and steroid 21-hydroxylation was reported.\textsuperscript{46, 54)} It is
strongly believed that CO-binding pigment is responsible for activation of oxygen in hydroxylation from the fact that this pigment in reduced form quickly reacts to oxygen and that hydroxylation is inhibited by CO and from its properties to recover photochemically from inhibited state and lastly from correlation between the induced synthesis of CO-binding pigment by injection of phenobarbitar and intensification of hydroxylation. As stated before, CO-binding pigment is more densely distributed in mitochondria than in microsomes of adrenals. The substance can be partly purified from acetone powder of mitochondrial fraction by Naja-Naja venom treatment. Of the sample thus obtained, absorption maxima are exhibited 415, 530 -- 540 m\(\mu\) in the oxidized form, at 421, 530 and 560 m\(\mu\) in the reduced, and reduced CO-binding at 421, 538, 560-570 m\(\mu\) all of which coincide with the results of the liver microsomal sample. By simultaneous addition of NADPH, NADPH dehydrogenase and adrenodoxin to the solublized CO-binding pigment, its enzymatic reduction in the presence of CO and under the anaerobic conditions may be achieved. (Figure-11)

Figure-11. Recomposition of Enzymatic Reduction System of Solublized CO-binding Pigment
** Absorbancy

** Wavelength

*** Solublized Co-binding pigment (P420), NADPH dehydrogenase and adrenodoxin were placed in the main section while NADPH was put in the side. After adding CO to the filled Kyubet (cuvette) under anaerobic conditions, and on addition of NADPH, reduction of CO-binding pigment was spectrally recorded by autographic device at a given time interval. The inserted figure is difference spectra of oxidized CO-binding pigment and reduced CO-binding. The spectra appeared when adrenodoxin was in presence. HS stands for Na₂S₂O₄ in chemically reduced form. (Kimura: Unpublished)

All the components used for the above experiment were in the solublized form and were at least partly purified. The electron transfer system was thus recomposed.

\[
\text{NADPH} \rightarrow \text{flavoprotein} \rightarrow \text{non-heme iron protein} \rightarrow \text{hemoprotein} \rightarrow O_2
\]
The arrows indicate the direction of electron flow which essentially agrees with that proposed by Omura et al.\textsuperscript{46} However, we hold reservation on decisive participation of CO-binding pigment in seroid hydroxylation because we assume reoxidation pathway by reduced non-heme iron protein. In other words, while it is found necessary for adrenal 11β-hydroxylation to add the fraction containing CO-binding pigment, one cannot positively prove that CO-binding pigment does participate in hydroxylation.

**Hydroxylation Position Determining Enzyme:**

For steroid hydroxylation, another species of enzyme which actually acts to incorporate activated oxygen into steroid particles should exist besides the electron transfer system from NADPH. Notwithstanding high similarities found in properties of electron transfer systems of hydroxylase complex, positions of hydroxylation indicate high specificity of substrates for 20α, 22, 11α and 18 etc. For example, at 11α, bond being axial, it is not easy to incorporate oxygen atom due to steric hindrance that exists for 10, 13 methyl radicals. Since steric hindrance at 11α is smaller, it may be assumed that oxygen atom enter through 11α first and then turn to 11β, nevertheless, this assumption has only gained negative results by experiments. Rather, 11α-T has been proved to become T+ on liberation by hydroxylation.\textsuperscript{56-58} It follows, therefore, that on liberation of 11β-H after becoming H+, activated oxygen OH+ carries out parental electron substitution. Although the presence of the enzyme system which enhance approach of oxygen
atom to steroid particles by slightly denting steroid nucleus and catalyze parental electron substitution might thus be assumed, its existence in reality has not yet been established.

**Summary**

Steroid hydroxylation may be classified into the following three stages according to the reactions:

1) $\text{NADPH} \rightarrow \text{NADP} + 2\text{H}^+ + 2\text{e}^{-}$

2) $\text{O}_2 + 2\text{H}^+ + 2\text{e}^{-} \rightarrow \text{OH}^- + \text{OH}^+$

3) $\text{OH}^+ + \text{AH} \rightarrow \text{H}^+ + \text{A}^\text{2-}\text{OH}$

Total: $\text{NADPH} + \text{O}_2 + \text{AH} \rightarrow \text{NADP} + \text{H}_2\text{O} + \text{A}^\text{-OH}$

For reaction 1) flavoenzymes are responsible and for 2) non-heme-iron protein, or non-heme iron protein and CO-binding pigment, and for 3) hydroxylation, position determining enzymes. These enzyme proteins are considered to catalyze hydroxylation of steroid by gathering as granules. In Figure-12, the author attempted to show a scheme of the enzymatic activities from functional aspect rather than microstructural point of view.

**Figure-12 Granular Diagram of Steroid Hydroxylases**
Oxygen, as becomes activated by electron transfer systems having common forms, would become fixed on the surface of the protein and then would be incorporated into a specific position of steroid particles for hydroxylation. One of the shortcomings of this diagram is to be found in its indication of the presence of such enzymes as pregnenolone (3β-OH dehydrogenase, 17α, 21-hydroxylase etc.) in microsomes and of not ending a series of enzyme system in the same granule. On the other hand, one may assume that steroid molecule tentatively dissociates from the granule on cleavage of side-chain with concomitant loss of hydrophilic properties.
have once increased on entrance of hydroxyl group from cholesterol.

It is interesting to note that the electron transfer system from NADPH of hydroxylation contains low molecular non-heme iron protein, particularly when we compare it with the electron transfer systems of other sources such as mitochondria, microsomes, chloroplasts and anaerobic bacteria. So far, non-heme iron protein in activated form has not been extracted from the electron transfer system of mitochondria (although Rieske et al.\textsuperscript{59} isolated low molecular non-heme iron protein from reduced CoQ-cytochrome \textit{c} reductase, it was denatured). On the grounds that there exist more than four non-heme iron in succinic dehydrogenase,\textsuperscript{13} and about 18 non-heme iron in NADH dehydrogenase\textsuperscript{60}, it may be surmised that non-heme iron protein of the electron transfer system in mitochondria is essentially similar to that in hydroxylation reaction in that both function as intermediates for electron transport between flavo enzyme and heme protein, although the latter is in simplified form. On the other hand, in microsomes, the system linked with cytochrome \textit{b}$_5$ is simple flavoprotein and non-heme iron is not present, which makes it different from the system in hydroxylation, however, to be of the same species as the system linked with CO-binding pigment. However, it must be mentioned here that there has never been a success report on isolation of low molecular non-heme iron protein from the fraction ascertained as microsomes and that the experiment using liver failed. Further,
significant amount of non-heme iron was found in purified CO-binding pigment but it is difficult to concluded that the system is quite identical to that in hydroxylation reaction. Among the electron transfer system of photosynthetic type, NADP reductase complex by PPNR (or ferredoxin) reduced by lighted induced chlorophyl also is a combination of non-heme iron protein and flavo enzymes. Except for its reverse electron flow, NADP reductase may even be considered as the same species as that belonging to hydroxylase complex. Similarity could also be sought with the electron transfer systems of nitrogen fixation reaction by anaerobic bacteria which is quite similar to the photosynthetic system and with the electron transfer system of hydrogen and CO₂ fixation which is a reverse reaction of phosphoclastic reaction. Further, studies on the electron transfer system from NADPH in hydroxylation reaction are closely related with observations on the systems of such oxygenases as pyrocatechase, metapyrocatechase, 3,4-dihydroxyphenyl acetate-2, 3- oxygenase containing only non-heme iron, salicylic hydroxylase containing flavin, p-oxybenzoic hydroxylase containing both flavin and non-heme iron, and tryptophanpyrrolease containing hematine in that they have similarity in fixation reactions within the living substance of such molecular gases as O₂, H₂, N₂. Viewed in conjunction with evolution in utilization of O₂ by living thing, these facts might give suggestions for evaluation of hydroxylation reaction from evolitional standpoint.
Meanwhile, because of too much resemblance in the electron transfer system of hydroxylases in adrenal mitochondria and in that of microsomes, there arise a question as to the inner cellular source of steroid hydroxylases. Generally, there is a tendency to show a marked growth in tubular smooth surface follicles in steroid hormone generating organs. Since there are some enzymes known to exist in microsomes among those which catalyze biosynthesis of steroid hormones, it might be possible to infer that all these hydroxylases are present in microsomes. However, the mitochondrial fraction obtained through fractionation of cellular granules, in which presence of hydroxylases were identified, showed high percentage of purity in the results of chemical analysis as well as of electron microscopic analysis. From the facts that indicate marked ups and downs in adrenal cortical mitochondria by removal of pituitary gland or by injection of ACTH, it might be possible to surmise that in steroid hormone synthesis, both mitochondria and microsomes contribute combined effects. In view of high metabolic control under which the adrenal cortical biosynthesis of corticoid and androgen is functioned, adrenal cortex is considered to have its cellular polarity besides zonal structure. Further, overwhelmingly strong NADPH generating system found in the soluble fraction is interpreted to limit the rate of NADPH membrane permeability thus lending the secondary control over metabolism. However, all these points remain to be studied further along with a
necessity to better understand acceleration effects which peptide hormones (ACTH) and cyclic AMP are reported to bring about in adrenal cortex.
Acknowledgement

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