HYDROXYLATION OF STEROIDS AT CARBON 21*

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Hydroxylation of steroids *in vitro* at carbon atom 21 by adrenal tissue preparations has previously been reported by Plager and Samuels (1), Hayano and Dorfman (2), and the present authors (3). The former workers described a system consisting of a 20,000 × *g* supernatant fraction of beef adrenals which effected hydroxylation in this position of progesterone and its derivatives in the presence of adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN).

The present work defines steroid C-21 hydroxylation as a reaction involving the microsomal fraction of beef adrenals, reduced triphosphopyridine nucleotide (TPNH), and atmospheric oxygen. Under these conditions progesterone, 11β-hydroxyprogesterone, 17α-hydroxyprogesterone, and 11,17-dihydroxyprogesterone are converted to their respective C-21 hydroxylated derivatives. With the concept of progesterone as a key intermediate in adrenal cortical hormone biogenesis (4), C-21 hydroxylation has the significance of an essential step in the synthesis of steroid hormones by this gland.

Materials and Methods

Tissue Preparation—Beef adrenal glands were collected in ice at the slaughterhouse, stripped of their capsules, and ground in a Waring blendor at 4° in a solution of 0.25 M sucrose, 0.1 M phosphate buffer, pH 6.8, and 0.04 M nicotinamide. In early experiments, the Krebs-phosphate buffer or 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer with 0.003 M $MgCl_2$ was used, and small quantities of tissue were ground with a Potter-

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Elvehjem glass homogenizer. These variations in buffer and tissue preparation had no effect on the results obtained. Cell fractions were separated by differential centrifugation as described by Schneider and Hogeboom (5). The microsomal fraction was sedimented between 15,000 × *g* and 105,000 × *g*, washed with a 0.25 M sucrose solution, and centrifuged. The 105,000 × *g* supernatant fraction was further purified by discarding protein which was precipitated from it at pH 5. Phase contrast microscopy of the mitochondrial and microsomal particles gave results similar to those reported for corresponding liver cell fractions (6). The protein content of enzyme preparations was measured by a trichloroacetic acid (TCA) turbidimetric method, standardized against dry weights after TCA and lipide solvent treatment (7).

The steroids used were commercial preparations which were chromatographed in solvent systems slightly modified from Bush's description (8) (see below) and found to be free from interfering contaminants which show ultraviolet absorption and reduce blue tetrazolium. TPNH was prepared by the method of Kaplan, Colowick, and Neufeld (9). DPNH, cytochrome *c*, and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Company. Crystalline catalase and ribonuclease were preparations of the Worthington Biochemical Corporation. Other cofactors, inhibitors, and substrates were also obtained from commercial sources.

Enzymatic Studies—Incubations were carried out in open 50 ml. Erlenmeyer flasks with constant agitation in a Warburg bath at 37° with air as the usual gas phase. Standard manometric techniques were employed for measuring oxygen uptake. Steroids dissolved in 0.1 ml. of propylene glycol or ethanol were added to mixtures containing tissue fractions and cofactors in a total volume of 3 to 5.3 ml. For studies on carbon monoxide inhibition, carbon monoxide and oxygen were mixed at constant pressure in a graduated cylinder, and used to displace the air in a Warburg vessel. Parallel controls with nitrogen and oxygen were always performed.

Extraction Procedures—The reaction mixtures were extracted twice with 4 volumes of methylene chloride; the extracts were pooled, washed with a 0.25 volume of saturated sodium bicarbonate solution, and taken to dryness *in vacuo*. The residue was dissolved in 20 ml. of 95 per cent methanol and extracted with an equal volume of hexane. The aqueous methanol fraction was evaporated under reduced pressure, and the residue dissolved in 2 ml. of 95 per cent ethanol.

Steroid Assay and Identification—The ethanol solution was assayed directly by the Porter-Silber method (10) by means of which the production of 17,21-dihydroxyprogesterone from 17-hydroxyprogesterone could be followed. Aliquots and reference standards were chromatographed in parallel by Bush's procedure (8) at 37° on Whatman No. 2 paper with the upper phase of modified solvent systems consisting of ligroin-toluene

(2:1 v/v) equilibrated with 70 per cent methanol-30 per cent water, and toluene equilibrated with 75 per cent methanol-25 per cent water. Ultraviolet absorption and reduction of blue tetrazolium were used to locate steroids. Their mobilities were compared with mobilities of known standards on the same chromatograms. Ethanol extracts were also pooled and subjected to a 99 transfer distribution in the Craig glass countercurrent apparatus (11). The contents of alternate tubes were analyzed by ultraviolet absorption at 240 mμ and by the development of color with the Porter-Silber reagent. Unchanged starting material and the product were separated and characterized by their partition coefficients. Good agreement of experimental and theoretical distribution curves was observed. Peak tubes of the product were pooled, and final identification of 17,21-dihydroxyprogesterone was made by infrared spectroscopy[1] with a Perkin-Elmer model No. 21 double beam instrument with sodium chloride optics. The Coleman junior spectrophotometer was used for measuring the sulfuric acid-phenylhydrazine chromogen of Porter and Silber at 410 mμ. TNPH oxidation was followed at 340 mμ in the Beckman DU spectrophotometer, and spectra of the cytochromes were determined in the Cary recording apparatus.

Results

In agreement with previous studies (1, 2), incubation of 17α-hydroxyprogesterone with adrenal gland preparations yielded 17,21-dihydroxyprogesterone (Reichstein's Substance S). This conversion is the model C-21 hydroxylation reaction which was followed in a majority of the studies described below.

Control Experiments—No detectable C-21 hydroxylation was observed when the complete system was extracted at zero time or when a suitable steroid substrate was omitted. Similar results were obtained with boiled enzyme and in the absence of enzyme. Recovery of added Substance S from the system at zero time was 89 per cent. Over the period of incubation, less than 10 per cent of this material was destroyed. The extraction procedure and assays proved to be reproducible, and agreement of duplicate runs was within 5 per cent. The data presented in Tables I to V represent typical experimental results based upon two or more replications for each experiment with quantitative and qualitative agreement.

Localization of Enzymatic Activity—As reported by Plager and Samuels (1), the C-21 hydroxylase activity of adrenal tissue was located in the 15,000 × *g* supernatant fraction, and the 11β-hydroxylase activity of the mitochondrial fraction (12, 13) could be completely separated from it. The rate of reaction and substrate concentration curves for the conversion

[1] Infrared spectroscopy was carried out by Mrs. D. Wheeler.

of 17α-hydroxyprogesterone to Substance S by the adrenal 15,000 × *g* supernatant fluid are demonstrated in Figs. 1 and 2. A pH optimum was

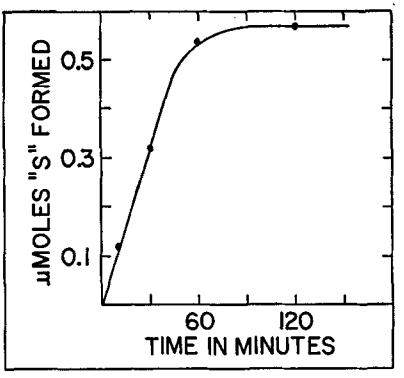

FIG. 1

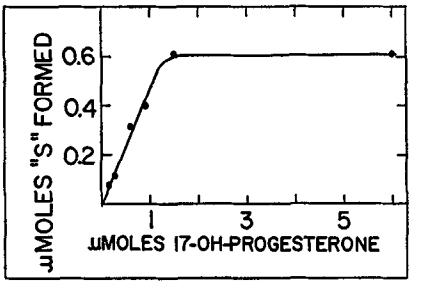

FIG. 2

FIG. 1. Conversion of 17-hydroxyprogesterone to 17,21-dihydroxyprogesterone (Reichstein's Substance S) as a function of time. The conditions were as follows: 15,000 × *g* beef adrenal supernatant fluid equivalent to 4 gm. of wet weight of tissue and the addition in micromoles per ml. of ATP 2, nicotinamide 5, DPN 0.5, and 17-hydroxyprogesterone 1.2, in a total of 5.3 ml. of Krebs-phosphate buffer at pH 7, incubated at 37°.

FIG. 2. Formation of Reichstein's Substance S as a function of substrate concentration. The conditions were the same as those described for Fig. 1, except for the concentration of 17-hydroxyprogesterone, which was varied, and an incubation time of 2 hours.

TABLE I

Requirement of Both Adrenal Microsome and Soluble Fractions for C-21 Hydroxylation

System*	Substance S formed
	μmole
15,000 × *g* supernatant	0.64
Microsomal fraction	0.00
105,000 × *g* supernatant (soluble fraction)	0.01
Recombination of microsomes and soluble fraction	0.59

* The system consisted of 15,000 × *g* supernatant fluid, 34 mg. of protein per ml., microsome fraction, 4 mg. of protein per ml., 105,000 × *g* supernatant fluid, 26 mg. of protein per ml., and, in micromoles per ml., phosphate buffer, 100, pH 6.8, ATP 0.5, nicotinamide 40, DPN 1, 17 α-hydroxyprogesterone 0.6, in a total of 5.3 ml. incubated at 37° for 1 hour.

exhibited between 6.5 and 7.0 with a loss of one-half of the maximal activity at 1 pH unit on either side of the optimum.

The C-21 hydroxylating activity of the 15,000 × *g* adrenal supernatant fluid could be separated into two components (105,000 × *g* supernatant and microsomal fractions) which were inactive when incubated alone but which were fully active upon recombination (Table I).

Rat liver 105,000 × *g* supernatant fluid could substitute for the corresponding adrenal fraction in recombination experiments with adrenal microsomes. Rat liver microsomes were inactive in recombination experiments.

Cofactor Requirements—Plager and Samuels demonstrated a DPN requirement of steroid C-21 hydroxylation and also an increased activity

TABLE II

Demonstration of Cofactor Requirement for C-21 Hydroxylation by Anion Exchange Resin Treatment of 105,000 × g Supernatant Fraction

Addition*		Substance S formed
	μmoles per ml.	*μmole*
None		0.00
ATP	2	0.00
DPN	1	0.24
DPNH	1	0.26
ATP	2	0.50
DPN	1	
TPN	1	0.38

* The system consisted of microsomal fraction, 4 mg. of protein per ml. recombined with 105,000 × *g* supernatant fluid, 25 mg. of protein per ml. The supernatant fraction was treated with 500 mg. per ml. of Dowex 1 (acetate form) prior to recombination. The following were added in micromoles per ml.: Tris buffer 50, pH 7.2, nicotinamide 40, and 17α-hydroxyprogesterone 0.6, in a total volume of 5.3 ml., incubated at 37° for 1 hour.

upon addition of ATP (14). In agreement with this, nicotinamide, ATP, and DPN were required in these experiments for maximal hydroxylating activity. If the 105,000 × *g* supernatant fluid was treated with an anion exchange resin (Dowex 1 (acetate form)), the recombined system of microsomes and supernatant fluid was inactive unless a pyridine nucleotide was added. ATP and DPN, or TPN alone, were more active than DPN or DPNH (Table II). The 105,000 × *g* supernatant fluid was also inactivated by being heated at 60° or 100° for 5 minutes. These data suggested that this fraction was contributing both an enzyme and a cofactor to the system.

A TPNH-generating system, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, and TPN in catalytic amounts, could replace the

105,000 × *g* supernatant fraction completely. TPNH in substrate amounts had the same effect. DPNH was only one-fifth as active as the reduced triphosphopyridine nucleotide, and ascorbate had no activity (Table III). Glucose-6-phosphate dehydrogenase was apparently already present in the microsome fraction, since glucose-6-phosphate and TPN were active without its addition (Table III). The relation of TPNH and microsomal protein concentration to C-21 hydroxylation are demonstrated in Figs. 3 and 4.

TABLE III

Requirement of TPNH for Steroid C-21 Hydroxylation by Adrenal Microsomes

Enzyme and substrate addition*	Cofactor		S formed
		μmoles per ml.	*μmoles*
105,000 × *g* supernatant	TPN	0.3	0.87
Glucose-6-phosphate dehydrogenase and glucose-6-phosphate	"	0.3	0.87
" "	No TPN		0.00
Glucose-6-phosphate	TPN	0.3	0.98
No glucose-6-phosphate	"	0.3	0.00
" "	TPNH	0.3	0.26
" "	"	3.0	1.41
" "	DPNH	3.0	0.26
" "	Ascorbate	10.0	0.00

* The system consisted of beef adrenal microsomal fraction, 6.3 mg. of protein per ml., and in micromoles per ml., Tris buffer 50, pH 7.2, nicotinamide 40, and 17α-hydroxyprogesterone 1.1, incubated in a total of 3 ml. at 37° for 1 hour.

TPNH oxidation was followed spectrophotometrically at 340 mμ, but the turbidity of the enzyme preparation and high rate of oxidation in controls without substrate precluded its use in following the reaction quantitatively.

Oxygen Requirement—Oxygen was required for C-21 hydroxylation, but 100 per cent oxygen had no significant advantage over air as the gas phase. No hydroxylation took place in nitrogen. The complete system had a sizable oxygen uptake when measured manometrically, but the high rate in the absence of added substrate made this method unsatisfactory for following steroid oxidation.

Characteristics of Adrenal Microsomes—The 15,000 × *g* adrenal supernatant fraction and the microsome particles prepared from it could be frozen and stored at −10° for several weeks without loss of activity. Lyophilized preparations were also active. Attempts to obtain the activity of the microsome particles in solution with butanol, hypotonic solutions, alternate freezing and thawing, sonic vibration, and steapsin were unsuc-

cessful. Treatment with 1 per cent sodium deoxycholate produced an optically clear system, which was, however, devoid of C-21 hydroxylase

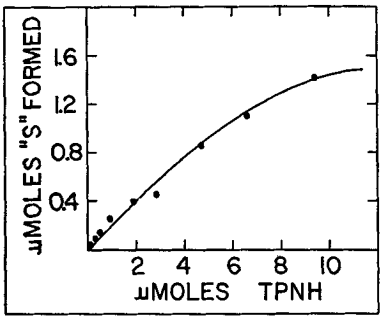

FIG. 3

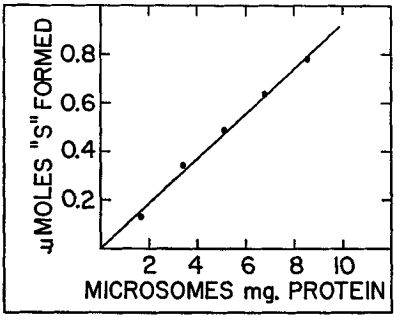

FIG. 4

FIG. 3. Formation of Reichstein's Substance S as a function of TPNH concentration. Conditions: Microsomal fraction, 6.3 mg. of protein per ml., and, in micromoles per ml., phosphate buffer 100, pH 6.8; nicotinamide 40, 17α-hydroxyprogesterone 1.1, in a total of 3 ml. incubated at 37° for 1 hour with variation in TPNH concentration as indicated.

FIG. 4. Formation of Reichstein's Substance S as a function of microsomal protein concentration. The conditions were the same as those described for Fig. 3 except for the concentration of microsomal protein, which was varied as indicated, and the concentration of TPNH, which was 1.5 μmoles per ml.

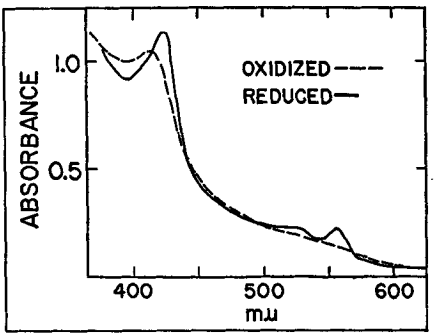

FIG. 5. Spectra of the cytochrome of microsome preparations equivalent to 25 mg. of protein made soluble with sodium deoxycholate. The spectra are tracings of the originals as recorded by a Cary spectrophotometer. The oxidized form with a maximum at 412 mμ represents the natural state of the cytochrome under the conditions of the preparation. The reduced form with maxima at 424, 522, and 557 mμ produced by treating the oxidized cytochrome with dithionite.

activity. Spectra of deoxycholate-treated microsome fractions had the characteristics of the cytochrome *m* described by Strittmatter and Ball (15) in liver microsome preparations (Fig. 5). Reduction of the cyto-

chrome was readily produced with TPNH and DPNH as well as with dithionite. Acetone powders of the microsomal fraction retained activity

TABLE IV

Effects of Inhibitors on C-21 Hydroxylation by Adrenal Microsomes and TPNH

Inhibitor classification*	Inhibitor	Concentration	Per cent of control activity
Sulfhydryl inhibitors	Iodoacetate	10^{-3} M	92
	o-Iodosobenzoate	10^{-3} "	95
	p-Chloromercuribenzoate	10^{-4} "	96
	"	10^{-3} "	1
	Mersalyl†	10^{-3} "	0
Heavy metals	Cupric sulfate	10^{-4} "	50
	Mercuric chloride	10^{-4} "	0
Reducing substances	Ascorbate	10^{-2} "	88
	Glutathione	10^{-2} "	112
Metal binders	EDTA‡	10^{-2} "	101
	Dipyridyl	10^{-3} "	94
Cytochrome inhibitors	Cyanide	10^{-3} "	86
	Azide	10^{-3} "	91
	Carbon monoxide, dark	90 per cent	35
	" " light	90 " "	57
Flavin inhibitor	Quinacrine	10^{-4} M	99
Miscellaneous	Antimycin A	1 γ per ml.	85
	Catalase	193 γ per ml.	100
	Ribonuclease	50 γ per ml.	96
	SKF 525 A§	10^{-3} M	91
	Cytochrome *c*	3×10^{-6} M	2
	" "	10^{-4} M	0
	" " + CN^-	10^{-3} "	95

Carbon monoxide experiments were carried out with a gas phase mixture of 90 per cent carbon monoxide and 10 per cent oxygen; control experiments with 90 per cent nitrogen and 10 per cent oxygen gave full activity.

* The system consisted of microsome fraction, 2.8 mg. of protein per ml. and, in micromoles per ml., phosphate buffer 100, pH 6.8, TPNH 0.9, 17α-hydroxyprogesterone 1.1, nicotinamide 40, in a total of 3 ml., incubated at 37° for 1 hour.

† Mersalyl (salyrgan), sodium *o*-((3-hydroxymercuri-2-methoxypropyl)carbamyl)-phenoxyacetic acid.

‡ EDTA (Versene), ethylenediaminetetraacetic acid.

§ SKF 525 A, diethylaminoethyldiphenylpropylacetic acid. (We are indebted to Dr. C. J. Kensler for a gift of this compound.)

in insoluble particles which could be sedimented at 105,000 $\times$ *g* in 1 hour. These particles contained 60 per cent of the cytochrome and 10 per cent of the protein of the intact microsome starting material.

Inhibitors—A wide variety of inhibitors, cofactors, and enzymes was

tested with the results shown in Table IV. The most dramatic effect was noted with cytochrome *c*, which produced 98 per cent inhibition at 3×10^{-6} M concentration. The inhibitory effect could be completely overcome with cyanide, suggesting that, under these conditions, the role of cytochrome *c* was that of a competitor for TPNH electrons.

A mixture of 90 per cent carbon monoxide and 10 per cent oxygen produced 65 per cent inhibition of C-21 hydroxylation which was partially reversed by light. There was no inhibition in control experiments in which a mixture of 90 per cent nitrogen and 10 per cent oxygen was employed. This light reversible carbon monoxide inhibition suggests the participation

TABLE V

Substrate Specificity of C-21 Hydroxylation

Substrate	Conversion product	By Porter-Silber reaction	Blue tetrazolium reduction
		μmole	
Progesterone	Deoxycorticosterone	0.00	+
11β-Hydroxyprogesterone	Corticosterone	0.06	+
17α-Hydroxyprogesterone	Substance S	0.66	+
11,17-Dihydroxyprogesterone	Cortisol	0.58	+
Δ⁵-Pregnen-3β-ol-20-one	Deoxycorticosterone	0.00	+
Δ⁵-Pregnene-3β,17α-diol-20-one	None	0.00	0

The system consisted of beef adrenal 15,000 × *g* supernatant fluid containing 36 mg. of protein per ml. with the following additions in micromoles per ml.: phosphate buffer 100, pH 6.8, DPN 0.5, ATP 2, nicotinamide 40, in 5.3 ml. of total volume. Incubated at 37° for 1 hour. All the products were identified by Bush paper chromatography by comparison with known standards. Δ⁵-Pregnene derivatives were incubated in a system similar to that described under Fig. 3. The conversion of Δ⁵-pregnen-3β-ol-20-one to deoxycorticosterone probably involved progesterone as an intermediate (see the text).

of the microsomal cytochrome in the hydroxylating system. No direct evidence for a cytochrome *m*-carbon monoxide complex is available, and Strittmatter and Ball reported no shift in the spectrum of the reduced cytochrome by this gas (15). Cytochrome *m* differs from cytochrome oxidase also in its lack of response to cyanide and azide (15).

Warburg first demonstrated light reversible inhibition of yeast cell respiration by gas mixtures of carbon monoxide and oxygen (16), and Keilin and Hartree described the carbon monoxide-induced shift in the absorption spectrum of reduced cytochrome oxidase (17). Subsequently, light reversible inhibition of enzymatic reactions has been one criterion for implicating the participation of iron-porphyrin-containing enzymes. How-

ever, there have been discrepancies between the effect of carbon monoxide on enzymatic reactions and its ability to produce shifts in spectra (16).

The results with other inhibitors were not sufficiently specific to draw conclusions about the mechanisms involved, although effects with mercurials and heavy metals suggested that sulfhydryl groups were important.

C-17 Hydroxylation—C-17 hydroxylation did not occur in most preparations tested. Progesterone was converted to deoxycorticosterone (21-hydroxyprogesterone) rather than to 17-hydroxyprogesterone or Substance S (Table V).

Substrate Specificity of Steroid C-21 Hydroxylase—Progesterone, 11β-hydroxyprogesterone, and 11,17-dihydroxyprogesterone were converted to their respective C-21 hydroxylated derivatives (Table V). Similar results were obtained with either the 15,000 $\times$ g supernatant fluid or the microsomal fraction with TPNH. Δ^5-Pregnen-3β,17α-diol-20-one was not acted upon, but Δ^5-pregnen-3β-ol-20-one was converted in very small yield to deoxycorticosterone, presumably by dehydrogenation to progesterone, followed by C-21 hydroxylation (Table V). A 3β-ol-dehydrogenase has been described (18) in microsome fractions, which converts Δ^5-pregnen-3β-ol-20-one to progesterone.

DISCUSSION

Steroid C-21 hydroxylation has thus been characterized as an oxidative reaction which requires adrenal microsomes, TPNH, and atmospheric oxygen. There are important similarities to other steroid hydroxylation systems and to many recently described oxidative reactions.

The oxygen for the hydroxyl group of the steroid C-11 (19), C-17, and C-21 (20) positions has been shown by O_2^{18} experiments to come directly from the atmosphere in adrenal and microbiological systems. Unfortunately the data for the C-21 position are based on work accomplished with microorganisms, and the existence of a similar pathway for the mammalian system can only be surmised. Although C-11 and C-21 hydroxylations occur in different cell fractions, the aspect of oxygen incorporation appears to be similar. Mason had previously demonstrated that the oxygen for phenol hydroxylation also comes directly from the atmosphere (21).

TPNH has been implicated as a cofactor requirement for all steroid hydroxylations thus far studied in detail (12), for a wide variety of oxidations of drugs (22), and for the conversion of lanosterol to cholesterol (23). Although it is not yet worked out, C-7 hydroxylation of bile acids appears to involve a similar system (24).

In addition to the present study involving adrenal microsomes with C-21 hydroxylation, the microsome fraction of liver has been implicated in the synthesis of cholesterol (6), bile acids (24), and the oxidation of drugs (22).

A concept of a general biological oxidation reaction can be evolved, which comprises the reactions discussed above and others even more widely distributed in nature (*cf.* Mitoma (25)), and which have the common features of the incorporation of atmospheric oxygen into the substrate and a relatively specific cofactor (TPNH, DPNH, and $FMNH_2$).[2] The oxygen transfer mechanisms for these unique oxidations are not yet known. Within this group of reactions, the soluble enzyme preparations involved in phenol (21) and phenylalanine oxidation (25) have been characterized as metalloproteins. One might also expect the specific steroid C-21 hydroxylating enzymes of the adrenal microsomal particles to be metalloproteins with heme or flavin moieties capable of accepting electrons from TPNH and catalyzing the introduction of oxygen into the substrate. Thus far, no metal or flavin requirement has been demonstrated for C-21 hydroxylation by inhibition studies (Table IV).

Hydrogen peroxide does not appear to be an intermediate (22) in view of the negative results with peroxide-generating systems (26) and the lack of inhibition with catalase (26) (Table IV), but the formation of organic peroxides has not been excluded.

Cytochrome *m*, which is present in large quantities in the microsomal fraction of liver and adrenal cells, can oxidize TPNH, DPNH, and ascorbate, and is itself autooxidizable (15). It has thus far not been possible to free the *active* C-21 hydroxylation system from this cytochrome by diverse means, nor has it been implicated directly in this or other reactions. In view of the large number of oxidative reactions now ascribable to the microsomal particles, it may be suggested that this cytochrome has an oxidative function rather than the reductive function originally proposed (15). Cytochrome *m* could function in the capacity proposed by Mason for the copper protein in phenol oxidation (21). In this case, the reduced cytochrome might form a complex with oxygen which could result in the oxidation of the steroid substrate. The light reversible carbon monoxide inhibition which we have observed lends support to such a hypothesis. In addition, however, an enzyme component must be present to provide the electron donor specificity and substrate specificity of the reaction.

SUMMARY

Steroid C-21 hydroxylation has been described as an oxidative reaction which occurs in beef adrenal microsomal preparations and requires TPNH and atmospheric oxygen. The reaction appears to be specific for progesterone and its derivatives. Similarities to other enzymatic reactions in which atmospheric oxygen is introduced into the substrates have been dis-

[2] $FMNH_2$, reduced flavin mononucleotide.

cussed, and a mechanism involving the microsomal cytochrome has been proposed.

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