PARTICIPATION OF CYTOCHROME b_5 ELECTRON TRANSPORT SYSTEM COUPLED WITH Δ^5 -3 β -HYDROXYSTEROID DEHYDROGENASE ON CYTOCHROME P-450 MONOOXYGENASE REACTIONS OF GUINEA PIG ADRENAL MICROSOMES*

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Not only cytochrome P-450 and cytochrome P-450 reductase, but considerable amount of cytochrome b_5 and cytochrome b_5 reductase were also contained in the adrenal microsomes of guinea pig.

Addition of nicotineamide adenine dinucleotide (NADH) stimulates the activities of cytochrome P-450 monooxygenases such as 21-hydroxylase, 17 α -hydroxylase and $C_{17,20}$ lyase supported with nicotineamide adenine dinucleotide phosphate (NADPH) as a cofactor. In addition, substrate for Δ^5 -3 β -hydroxysteroid dehydrogenase plus NAD^+ also stimulate the same cytochrome P-450 monooxygenase activities.

These observations were suggesting the participation of cytochrome b_5 electron transport system on cytochrome P-450 monooxygenase (21-hydroxylase, 17 α -hydroxylase and $C_{17,20}$ lyase) reactions associated with the biosynthesis of corticoids and adrenal androgens. Furthermore, it is also suggesting that cytochrome b_5 electron transport system involves the reaction of Δ^5 -3 β -hydroxysteroid dehydrogenase.

Keywords — adrenal microsome; cytochrome b_5 ; cytochrome P-450 monooxygenase; 21-hydroxylase; 17 α -hydroxylase; $C_{17,20}$ lyase; Δ^5 -3 β -hydroxysteroid dehydrogenase; corticoid; adrenal androgen

In the adrenal cortex, steroid metabolism involves a sequence of enzyme reactions located in the mitochondria and microsomes. It is well known that some of these enzymes are cytochromes P-450 monooxygenases.¹⁾ The cytochromes P-450 located in the microsomes catalyze 21-hydroxylation, 17 α -hydroxylation and cleavage of the $C_{17,20}$ bond of C_{21} steroids.¹⁾ The enzyme (P-450), which catalyzes 21-hydroxylation of progesterone and 17 α -hydroxyprogesterone has been purified from microsomes of pig²⁾ and bovine adrenal.³⁾ Recently, another cytochrome P-450 which catalyzes 17 α -hydroxylation and $C_{17,20}$ cleavage of C_{21} steroids was purified from the adrenal microsomes of pig⁴⁾ and guinea pig⁵⁾ as a single species of pro-

tein. Enzyme activities of these cytochromes P-450 were reconstituted with purified cytochrome P-450 and nicotineamide adenine dinucleotide phosphate (NADPH)-cytochrome P-450 reductase.

Some of the drug-metabolizing reactions catalyzed by cytochrome P-450 in liver microsomes, show increased activity on addition of nicotineamide adenine dinucleotide (NADH), which stimulates enzyme activity by providing the second electron of P-450 cycle *via* cytochrome b_5 and cytochrome b_5 reductase.⁶⁾ In addition, it has been suggested that electron transport system involving the cytochrome b_5 may participate in androgen biosynthesis by testicular microsomes.⁷⁾ Studies using a system reconstituted

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from purified components, strongly supported this suggestion.^{8,9)}

In this paper, we show that NADH stimulates 21-hydroxylation, 17 α -hydroxylation and C_{17,20} lyase activity by providing the second electron of the P-450 cycle *via* cytochrome b₅ and cytochrome b₅ reductase, in addition, NADH formed as a result of conversion of 3 β -hydroxy- Δ^5 -steroids (*i.e.* pregnenolone) to 3-keto- Δ^4 -steroid (*i.e.* progesterone) by Δ^5 -3- β -hydroxysteroid dehydrogenase with $\Delta^4,5$ -ketosteroid isomerase is used for the cytochrome b₅ electron transport system.

MATERIALS AND METHODS

Preparation of Adrenal Microsomes —

The microsomes were prepared from the adrenal glands of guinea pig (Hartley, male: 260–330 g, female: 270–320 g). Whole adrenal glands were homogenized with 0.15 M KCl–5 mM ethylenediaminetetra acetic acid (EDTA). The homogenate was centrifuged at 10 000 $\times g$ for 20 min, and the supernatant fluid was centrifuged at 78 000 $\times g$ for 90 min. The precipitate obtained was washed with 0.15 M KCl–5 mM EDTA and 0.1 M potassium phosphate (pH 7.4)–1 mM EDTA. The washed microsomes were stored in 20% (v/v)-glycerol–0.1 M potassium phosphate (pH 7.4)–0.1 mM EDTA at –80°C.

Enzyme Assay — Enzyme activities of 21-hydroxylase, 17 α -hydroxylase, C_{17,20} lyase and 3 β -hydroxysteroid dehydrogenase with isomerase were measured by incubation of radioactive substrate with the microsomes and cofactor. The microsomes from guinea pig adrenals were preincubated with 5 nmol radioactive steroid in 50 mM potassium phosphate (pH 7.4) for 3 min. [4-¹⁴C]-Progesterone (32000 cpm/5 nmol) and [4-¹⁴C]-17 α -hydroxyprogesterone (29000 cpm/5 nmol) were used as the substrate for 21-hydroxylase, 17 α -hydroxylase, and 21-hydroxylase, C_{17,20} lyase, respectively. [4-¹⁴C]-Pregnenolone, [4-¹⁴C]-17 α -hydroxy-pregnenolone and [4-¹⁴C]-dehydroepiandrosterone (30000 cpm/5 nmol each) were used as the sub-

strate for 3 β -hydroxysteroid dehydrogenase with isomerase. The reaction was started by adding 240 nmol of NADPH (or NAD⁺ for 3 β -hydroxysteroid dehydrogenase) for 5 min in 1 ml of incubation medium.

Following incubation, steroids were extracted with 10 ml of methylenechloride. The produced radioactive steroids were determined by liquid scintillation spectrophotometer following separation by thin layer chromatography as described previously.¹⁰⁾ The activity of 21-hydroxylase was expressed as sum of 11-deoxycorticosterone and 11-deoxycortisol and that of 17 α -hydroxylase was expressed as sum of 17 α -hydroxyprogesterone and 11-deoxycortisol. C_{17,20} lyase activity was expressed as amount of androstenedione produced, and the activity of 3 β -hydroxysteroid dehydrogenase plus isomerase was expressed as amount of 3-keto- Δ^4 -steroid produced from 3 β -hydroxy- Δ^5 -C₂₁steroid.

Content of cytochrome P-450 and cytochrome b₅ of adrenal microsomes, and NADH- and NADPH-ferricyanide reductase activities were measured by established method.^{11,12)}

Protein Determination — Protein content was determined according to the method of Lowry *et al.*¹³⁾ with bovine serum albumin as the standard.

Chemicals — Radioactive steroids were purchased from New England Nuclear Corp., Boston. Non-radioactive steroids, NAD⁺, NADH, NADPH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co., St. Louis. All of other reagents used were of the best commercially available grade.

RESULTS AND DISCUSSION

Electron Carriers and Enzymes in Microsomes from Guinea Pig Adrenals

As shown in Table I, the microsomes from guinea pig adrenals contain cytochrome b₅ and cytochrome b₅ reductase (NADH-ferricyanide reductase) as described previously in cases of microsomes from rat⁷⁾ and neonatal pig testis.⁸⁾ It

TABLE I. *Electron Carrier and Enzymes in Microsomes from Guinea Pig Adrenal*

Iterum	Male		Female	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Cytochrome P-450 ^{a)}	2.65	2.41	3.00	2.61
Cytochrome b_5 ^{a)}	1.42	1.44	1.61	1.37
NADPH-ferricyanide reductase ^{b)}	0.51	0.53	0.49	0.52
NADH-ferricyanide reductase ^{b)}	2.98	2.86	2.79	3.21

a) nmol/mg protein, b) μ mol/min/mg protein.

The microsomes were prepared from adrenal glands of 5 animals in each experiment, and electron carrier and enzymes were measured in microsomes as described in Materials and Methods.

should be noticed that there were considerable amounts of cytochrome b_5 and cytochrome b_5 reductase in adrenal microsomes of guinea pig as in testicular microsomes of neonatal pig, and there was no difference between the microsomal content of these enzymes from male and female animals.

Steroid-Metabolizing Enzymes in Adrenal Microsomes

Fig. 1 shows a time-course of metabolism of progesterone incubated with microsomes from guinea pig adrenals and NADPH. Levels of 11-deoxycortisol and androstenedione increased with time. 17 α -Hydroxyprogesterone and 11-deoxycorticosterone accumulated transiently, reaching a maximum and then gradually decreasing. The above observations indicated that 17 α -hydroxyprogesterone was the major intermediate from progesterone to 11-deoxycortisol or androstenedione during the biosynthesis of corticoids and adrenal androgen, respectively.

Table II shows 21-hydroxylase, 17 α -hydroxylase, C_{17,20} lyase and 3 β -hydroxysteroid dehydrogenase with isomerase activities when substrate was incubated with microsomes from guinea pig adrenals. It was recognized that the microsomes catalyzed the conversion of 3 β -hydroxy- Δ^5 -steroids to 3-keto- Δ^4 -steroids with pregnenolone, 17 α -hydroxypregnenolone and dehydroepiandrosterone as substrates. In addition, there was no difference between male and female microsomes in the reaction and relative

FIG. 1. *Metabolism of Progesterone by Microsomes from Guinea Pig Adrenal*

Adrenal microsomes (50 μ g) were incubated with [4- 14 C]-progesterone (5 nmol/4 $\times 10^4$ cpm) with glucose 6-phosphate (5 μ mol), glucose 6-phosphate dehydrogenase (1 unit), MgCl₂ (0.5 μ mol), NADPH (240 nmol) and potassium phosphate (50 mM, pH 7.4) to a total volume of 1.0 ml. Following incubation, steroids were extracted with methylene chloride and were separated by thin layer chromatography in the systems ethylacetate-hexane (3:7, v/v). Steroids were identified and 14 C was measured. Progesterone (Δ), 17 α -hydroxyprogesterone ($\circ$), 11-deoxycorticosterone ($\bullet$), 11-deoxycortisol ($\square$), androstenedione ($\blacksquare$).

TABLE II. *Enzyme Activities of Steroid-Metabolizing Enzymes in Microsomes from Guinea Pig Adrenal*

Steroid-Metabolizing enzyme	Substrate	Enzyme activity (nmol/min/mg protein)			
		Male		Female	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
21-Hydroxylase	Progesterone	5.8	5.5	6.6	5.3
	17 α -Hydroxyprogesterone	7.0	7.5	9.0	6.7
17 α -Hydroxylase	Progesterone	9.4	9.2	9.8	9.7
C _{17,20} Lyase	17 α -Hydroxyprogesterone	4.5	5.7	4.8	5.2
3 β -Hydroxysteroid dehydrogenase with isomerase	Pregnenolone	14.0	13.7	12.6	14.3
	17 α -Hydroxypregnenolone	10.7	—	9.9	—
	Dehydroepiandrosterone	20.5	—	19.2	—

The microsomes were prepared from adrenal glands of 5 animals in each experiment, and enzyme activities were measured in microsomes as described in Materials and Methods.

rates observed for these reactions.

Effect of NADH on 17 α -Hydroxylase, C_{17,20} Lyase and 21-Hydroxylase Activities

As shown in Fig. 2, the addition of NADH to 17 α -hydroxylation, C_{17,20} cleavage and 21-hydroxylation system which were supported by NADPH caused the enzyme activity to be significantly increased at low concentrations (5×10^{-6} – 1×10^{-4} M) of an equal mixture of two pyridine nucleotides. This suggested that steroid-metabolizing enzymes, such as 21-hydroxylase, 17 α -hydroxylase and C_{17,20} lyase, which were involved in the conversion of progesterone to corticoids and adrenal androgens by microsomes would use cytochrome b₅ as the source of the second electron from NADPH to cytochrome P-450 *via* cytochrome b₅ reductase and cytochrome b₅.

Effect of 3 β -Hydroxysteroid Dehydrogenase Reaction on 21-Hydroxylase, 17 α -Hydroxylase and C_{17,20} Lyase Activities

Table III shows the effect of 3 β -hydroxysteroid dehydrogenase reaction on the activities of microsomal cytochrome P-450 monooxygenases (21-hydroxylase, 17 α -hydroxylase and C_{17,20} lyase). By addition of dehydroepiandrosterone and NAD⁺ as the substrate and as the cofactor for 3 β -hydroxysteroid dehydrogenase, re-

spectively, the enzyme activities of 21-hydroxylase, 17 α -hydroxylase and C_{17,20} lyase which were supported with NADPH were significantly enhanced.

In testicular microsomes, it has been shown that microsomal cytochrome b₅ was reduced by addition of pregnenolone, and that C_{17,20} lyase activity supported by NADPH was enhanced by addition of dehydroepiandrosterone and NAD⁺.⁷⁾ In addition, it has also been shown that cytochrome b₅ in the adrenal microsomes of guinea pig was reduced by addition of pregnenolone and NAD⁺.¹⁴⁾ We have shown that not only pregnenolone, but also dehydroepiandrosterone reduced cytochrome b₅ in the presence of NAD⁺ (data not shown).

In conclusion, three lines of evidence suggest that electron transport *via* cytochrome b₅ from 3 β -hydroxysteroid dehydrogenase may participate in the synthesis of corticoids and adrenal androgens by adrenal microsomes: (1) There are considerable amounts of cytochrome b₅ and cytochrome b₅ reductase in adrenal microsomes. (2) Addition of NADH stimulates the activities of these enzymes such as 21-hydroxylase, 17 α -hydroxylase and C_{17,20} lyase. (3) Substrate for 3 β -hydroxysteroid dehydrogenase plus NAD⁺ also stimulate the same cytochrome

FIG. 2. Effect of Cofactor on Activities of 17 α -Hydroxylase, C_{17,20} Lyase and 21-Hydroxylase
 Adrenal microsomes (22 μ g) were incubated with radioactive substrate in the presence of pyridine nucleotide shown, NADH (●), NADPH (○) and both of NADH and NADPH (●) in 1 ml of 50 mM potassium phosphate (pH 7.4) for 5 min at 37°C.
 A: 17 α -hydroxylase activity, B: C_{17,20} lyase activity, C: 21-hydroxylase activity of 17 α -hydroxyprogesterone, D: 21-hydroxylase activity of progesterone.

TABLE III. Effect of Reaction by Δ^5 -3 β -Hydroxysteroid Dehydrogenase on 17 α -Hydroxylase, 21-Hydroxylase and C_{17,20} Lyase Activities

Enzyme	Addition	Enzyme activities	
		(nmol/min/mg protein)	(%)
17 α -Hydroxylase	Control	8.6 $\pm$ 0.14 ^{a)}	100
	+NAD ⁺ (100 μ M)	10.9 $\pm$ 0.33 ^{b)}	126
21-Hydroxylase	Control	5.4 $\pm$ 0.40	100
	+NAD ⁺ (100 μ M)	7.3 $\pm$ 0.29 ^{b)}	135
C _{17,20} Lyase	Control	4.6 $\pm$ 0.13	100
	+NAD ⁺ (100 μ M)	5.6 $\pm$ 0.20 ^{b)}	121

The microsomes (22 μ g protein) from guinea pig adrenal were incubated with 5 nmol radioactive substrate in 1 ml of 50 mM potassium phosphate (pH 7.4) for 5 min at 37°C. Control assay mixture was contained NADPH (100 μ M) and non-radioactive dehydroepiandrosterone (50 μ M).

a) Mean $\pm$ S.D. b) $p < 0.05$.

P-450 monooxygenases (presumable by supplying NADH).

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