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SUBMICROSOMAL DISTRIBUTION OF ADRENAL ENZYMES AND CYTOCHROME P-450 RELATED TO CORTICOIDOGENESIS

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SUMMARY

By density-gradient centrifugation in the absence of CsCl, the microsomal fractions obtained from porcine and rat adrenal glands were separated into two subfractions: one fraction consisted of smooth-surfaced membrane structures which bore no ribosomes on their outer surface, and the other consisted of abundant free ribosomes with membrane structures, according to electron microscopic observation. The activities of three microsomal enzymes related to corticoidogenesis, namely Δ^5-3β-hydroxysteroid dehydrogenase (EC 1.1.1.51) coupled with the Δ^5–Δ^4 isomerase (EC 5.3.3.1), 17α-hydroxylase (EC 1.14.1.7) and 21-hydroxylase (EC 1.14.1.8), were predominantly located in the smooth-surfaced subfraction. In accordance with the distribution of the hydroxylases, adrenal microsomal cytochrome P-450 was concentrated exclusively in the smooth-surfaced subfraction. As 21-hydroxylation was significantly inhibited in a CO-containing atmosphere, the cytochrome P-450 in the smooth-surfaced subfraction was participating as the direct activating site of O_2 during 21-hydroxylation.

The K_m values of the submicrosomal Δ^5-3β-hydroxysteroid dehydrogenase for pregnenolone and of 17α-hydroxylase for progresterone were estimated as 0.2 and 0.13 mM, respectively. The K_m of the adrenal 21-hydroxylase was evaluated as 99 μM for progesterone and as 0.22 mM for 17α-hydroxyprogesterone. Substrate specificities of the adrenal submicrosomal enzymes, dynamic analysis of the corticoidogenesis throughout the incubation time, and the relationship between the amounts of enzyme preparations and the products, were examined.

INTRODUCTION

Since the subfractionation of the hepatic microsomal fraction into rough- and smooth-surfaced membrane structures by density-gradient centrifugation, it has been reported that some drug-metabolizing enzymes[1,2] and the enzyme systems related to cholesterol biosynthesis from acetate[3] are localized mainly in the hepatic smooth-surfaced subfraction.

On the other hand, well-developed smooth-surfaced endoplasmic reticula have been observed in the steroid-producing cells of tissues such as the adrenal cortex[4], corpus luteum[5] and testicular interstitial tissue[6]. In our preliminary communication[7],

the activities of the Δ^5-3β-hydroxysteroid dehydrogenase and 21-hydroxylase of rat adrenal glands were found mostly in the smooth-surfaced subfraction.

This paper deals with the detailed analysis of the submicrosomal distribution of the enzyme activities related to corticoidogenesis, the cytochrome P-450 of adrenal microsomal fractions of pig and rat, the role of the cytochrome P-450 in adrenal microsomal hydroxylations, and enzymological properties including kinetic studies of the submicrosomal preparation.

MATERIALS AND METHODS

Tissue preparations

After male rats of the Wistar strain (3 month old) had been decapitated, the adrenal glands were isolated and decapsulated. Pooled adrenals were weighed and homogenized with a loose-fitting glass-Teflon homogenizer in an ice-cold 0.33 M sucrose solution buffered with 0.05 M Tris–HCl buffer (pH 7.4) and 0.005 M $MgCl_2$. After the centrifugation of the homogenates at $800 \times g$ for 20 min, the supernatant fluid was centrifuged at $6000 \times g$ for 20 min, and the precipitate thus obtained was used as the mitochondrial fraction. After the supernatant fluid obtained at $6000 \times g$ was centrifuged at $10\,000 \times g$ for 20 min, the precipitates which contained the light mitochondrial fraction were discarded. The subsequent supernatant fluid was centrifuged at $105\,000 \times g$ for 60 min and the precipitate was resuspended in 0.33 M sucrose solution and used as the microsomal fraction. For further submicrosomal separation, 3.0 ml of the microsomal suspension obtained at $105\,000 \times g$ was layered over 7.0 ml of 1.23 M sucrose solution in centrifugation tubes. After centrifugation at $105\,000 \times g$ for 18 h, a compact dark brown pellet was obtained at the bottom of the tubes, while a light brown diffuse layer was found where the boundary of the two phases had been before the centrifugation. The diffuse layer of the submicrosomal fraction was aspirated with a J-shaped transfer pipette, diluted with 0.33 M sucrose solution, and recentrifuged at $105\,000 \times g$ for 60 min. The precipitate thus obtained was suspended in the 0.33 M sucrose solution, and this suspension is referred to in this paper as the smooth-surfaced subfraction. After the remaining sucrose solution had been removed, the compact pellet was obtained from the bottom of the tubes, and this subfraction was named the rough-surfaced subfraction following the conventional terminology of hepatic subfractionation. These two subfractions were subjected to enzyme assays, measurement of cytochrome P-450, and also to electron-microscopic examination.

Porcine adrenals were obtained from the Keiyo Ham Co. (Chiba), and brought to the laboratory on ice. Preparation of the adrenal microsomal fraction of pig and subfractionation of the microsomal fraction followed essentially the same procedure as that for the rat adrenal microsomal fraction.

Radioactive steroids

[4-^{14}C]Pregnenolone (specific activity, 758.3 μC/mg) and 17α-[4-^{14}C]hydroxyprogesterone (specific activity, 113.6 μC/mg) were purchased from the Radiochemical Centre, Amersham, England. [4-^{14}C]Progesterone (specific activity, 146.5 μC/mg), 17α-[7α-^{3}H]hydroxypregnenolone (specific activity, 40.0 mC/mg), 11-[4-^{14}C]deoxycorticosterone (specific activity, 133.1 μC/mg) and [4-^{14}C]dehydroepiandrosterone (specific activity, 145.5 μC/mg) were purchased from New England Nuclear Corp.,

Boston, Mass. [4-^{14}C]Cholesterol (specific activity, 30.4 μC/mg) was purchased from the Dai-ichi Chemical Co., Tokyo. The radiochemical purity of these steroids was confirmed by thin-layer chromatography shortly before use. Radioactive steroids were diluted with the corresponding non-radioactive steroids to the appropriate specific activities for substrates for enzyme assays, and also to ensure saturation of the enzymes with each substrate.

Incubation

The radioactive steroids dissolved in volatile organic solvents were transferred to incubation flasks, into which 5 drops of propylene glycol per flask were added. Shortly before the incubation, the solvent was removed under reduced pressure. The mixture of enzyme preparation in 0.33 M sucrose solution containing 0.05 M Tris (adjusted with HCl to pH 7.4) and 0.005 M $MgCl_2$, and 1 ml of the 0.33 M sucrose solution which contained NADPH or NAD^+ (Sigma Chem. Co., St. Louis, Mo.), were then added to the flasks.

Extraction, isolation and identification of the metabolites

Immediately after incubation, enzyme reactions were arrested by the addition of 15 ml of methylene dichloride, and the mixture was vigorously shaken to extract the steroids. Then, after centrifugation of the mixture at 400 $\times g$ for 10 min, the lower layer was removed by a transfer pipette and retained. The above procedure was repeated two more times in order to complete the extraction of the steroids. The pooled extract was dehydrated with anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure. At this stage, 90–98% of the radioactivity initially added was recovered in the extract. An aliquot of the radioactive steroid extract was chromatographed on a thin-layer plate coated with silica gel G and GF (Merck, Darmstadt, Germany), using a benzene–acetone (8:2, by vol.) solvent system at 15–20°. After development of the thin-layer plate was complete, spots of the carrier steroids on the chromatogram were detected under ultraviolet light (wavelength, 253 mμ). Radioactive spots were detected by an autoradiographic method by exposing an X-ray film to the coated phase of the chromatogram for a week, and also by a windowless gas-flow counter with a scanning device (Vanguard Autoscanner 880 and 885, Technical Measurement Co., North Haven, Conn.). The spots of steroids detected were scraped off the thin-layer plate and extracted with the mixture of chloroform–ethyl alcohol (1:1, by vol.) from the absorbent which had been packed as a small column. Final identification of the metabolites obtained was based on the following criteria: (1) Identical mobilities of the metabolites to those of the corresponding standard preparations on thin-layer chromatograms developed with the different solvent systems. (2) Identical chromatographic behavior to those of the standard preparations after chemical reactions, such as oxidation by CrO_3 in aqueous acetic acid solution and acetylation with acetic anhydride in pyridine. (3) Constant specific activities of crystals obtained by repeated crystallization of the radioactive metabolites with the corresponding authentic preparations, as stated in our previous paper[8].

Quantitation of radioactivities of the products

Suitable aliquots of the extracts were evaporated to dryness under N_2 in 20-ml glass vials (Wheaton Glass Co., Millville, N.J.), and dissolved in 11 ml of toluene

solution which contained 2,5-diphenyloxazole (0.4%) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (0.01%). The radioactivity was then measured with a liquid scintillation spectrometer (System 725, Nuclear-Chicago, Des Plaines, Ill.) for a sufficient time to reduce the counting error to below 5%. The efficiencies of counting ^{14}C and ^{3}H were about 70 and 39%, respectively. From the specific activities of the administered substrate steroids, the amounts of the products were estimated in μmoles.

Assay of adrenal enzymic activities

The adrenal enzyme activities related to steroid bioconversion were expressed as follows; Δ^5-3β-hydroxysteroid dehydrogenase activity together with the Δ^5–Δ^4 isomerase was expressed as the sum of the Δ^4-3-oxosteroids formed from the incubated pregnenolone. 17α-Hydroxylase activity was expressed as the sum of the 17α-hydroxyprogesterone and 11-deoxycortisol formed from the progesterone, while the 21-hydroxylase activity for progesterone was defined as the sum of the 11-deoxycorticosterone and 11-deoxycortisol formed from progesterone. The 21-hydroxylase activity upon 17α-hydroxyprogesterone was expressed as the amount of 11-deoxycortisol derived from the 17α-hydroxyprogesterone.

Measurement of protein, RNA and cytochrome P-450

Protein concentrations of the enzyme preparations were determined by the copper–Folin method, using a 1-cm light path at 750 mμ, after diluting the samples to a suitable concentration[9]. Measurements of the RNA content of the microsomal and submicrosomal fractions were based on their ribose content using the method of CERIOTTI[10]. The cytochrome P-450 content in the smooth- and rough-surfaced submicrosomal fractions was estimated by essentially the same method as described by HART AND FOUTS[11], using a recording photospectrometer (Hitachi–Perkin–Elmer, EPS-3T) with a diffuse reflectance attachment.

RESULTS

Electron-microscopic observation and chemical analysis of the submicrosomal fractions of rat and porcine adrenal glands

Cytological observation of the adrenal subfractions with an electron microscope revealed that the smooth-surfaced fraction consisted mainly of smooth-surfaced membrane structures, and the other subfraction named the rough-surfaced subfraction was a mixture of a large quantity of free ribosomes with a limited amount of membrane structure, as shown in Fig. 1. It was also noticed that the microsomal fraction, obtained as the precipitate at 10 000–105 000 $\times g$, was not contaminated by mitochondria.

According to biochemical analysis of adrenal microsomal subfractions, the rough-surfaced microsomal fraction contained about 2–7 times as much RNA per unit weight of protein as the smooth-surfaced microsomal fraction, as shown in Table I. The rough-surfaced submicrosomal fraction of porcine adrenal showed a high ratio of RNA to protein, probably because of the abundant presence in the subfraction of free ribosomes whose ratio was 0.5, and because of the loss of some protein from the rough-surfaced microsomal fraction due to its solubilization by repeated washing with 0.154 M KCl solution, before the determination of RNA.

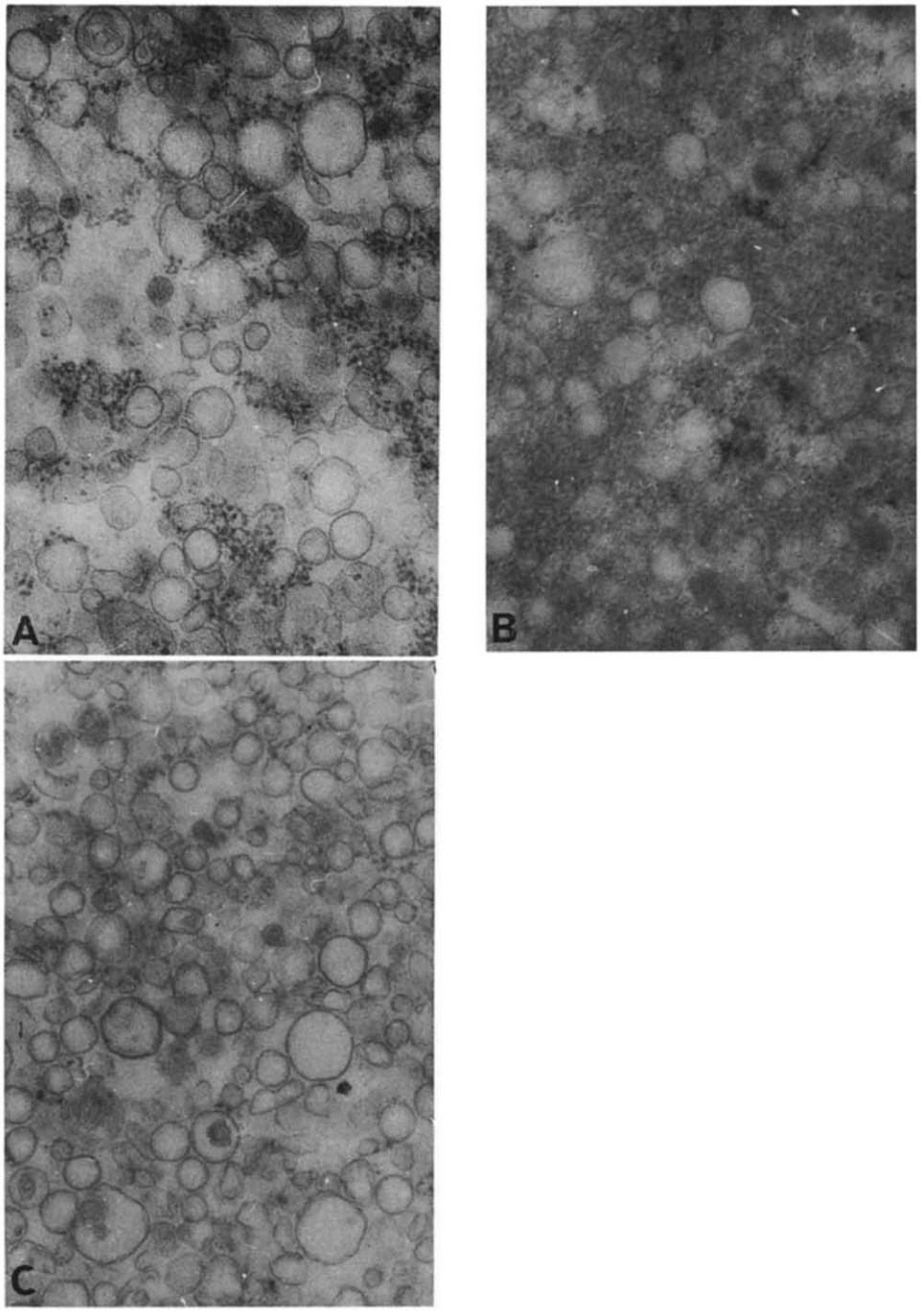

Fig. 1. Electron micrograph of the total microsomal fraction and its smooth- and rough-surfaced subfractions of porcine adrenals (× 60 000). A. Total microsomal fraction or precipitates after centrifugation at 105 000 × *g* for 60 min. Abundant smooth-surfaced vesicles and free ribosomes, but few rough-surfaced ones are observed. B. Rough-surfaced subfraction after density-gradient centrifugation. A large quantity of free ribosomes with the membrane structure was noted. C. The smooth-surfaced subfraction which bore no ribosomes on the outer surface.

TABLE I

PROTEIN AND RNA CONTENTS OF THE MICROSOMAL FRACTION AND ITS TWO SUBFRACTIONS OF PORCINE AND RAT ADRENAL GLANDS

Microsomal fractions, obtained from the homogenates of porcine adrenals (52 g wet wt.) and rat adrenals (5.1 g wet wt.) by differential centrifugation, were diluted respectively to 30.0 and 9.0 ml with 0.33 M sucrose solution. For further fractionation of the total microsomal fractions, the microsomal suspension (3.0 ml) was layered on 7.0 ml of 1.23 M sucrose solution per centrifugation tube. After centrifugation of these tubes at 105 000 × *g* for 18 h, smooth- and rough-surfaced subfractions were separated. After centrifugation of the isolated subfractions in 0.33 M sucrose solution at 105 000 × *g* for 60 min, the precipitate was suspended in 0.154 M KCl solution and then recentrifuged at 105 000 × *g* for 60 min. The procedure was repeated 3 times to remove the sucrose which had been used as solute. Protein and RNA concentrations were determined by the copper–Folin[9] and orcinol reactions[10], respectively. Before washing the precipitates with KCl solution, a part of the suspension of each subfraction was reserved for the study of the distribution of the enzyme activities and for measurement of cytochrome P-450.

Fraction	*Protein (mg/g adrenal)*		*RNA (mg/g adrenal)*		*RNA/protein ratio*	
	Pig	*Rat*	*Pig*	*Rat*	*Pig*	*Rat*
Total microsomal fraction	259	1068	102	174	0.39	0.16
Smooth-surfaced subfraction	71	604	17	36	0.24	0.06
Rough-surfaced subfraction	4	48	2	20	0.50	0.42

Submicrosomal distribution of the enzyme activities of the adrenal microsomal fraction related to steroidogenesis

After incubation of progesterone and pregnenolone with the two submicrosomal fractions, the metabolites were identified by the methods stated previously and quantitated. 11-Deoxycorticosterone, 17α-hydroxyprogesterone and 11-deoxycortisol were identified as the metabolites produced from progesterone by the smooth-surfaced microsomal fraction of porcine adrenals. These three compounds and progesterone were isolated and identified as the metabolites of pregnenolone. Using the smooth-surfaced submicrosomal preparation of rat adrenal, 11-deoxycorticosterone was identified as the metabolite of progesterone, and 11-deoxycorticosterone and progesterone were found to be the metabolites of pregnenolone.

From the results obtained by quantitative measurement of the metabolites, the enzyme activities of both subfractions were estimated. In the case of rat adrenal glands, the activities of Δ^5-3β-hydroxysteroid dehydrogenase and 21-hydroxylase were concentrated in the smooth-surfaced microsomal fraction (Table II). Also, in the case of porcine adrenal glands, the same submicrosomal distribution of Δ^5-3β-hydroxysteroid dehydrogenase and of the 17α- and 21-hydroxylases was observed, and their specific activities were about twice that of their activity in the total microsomal fraction. However, the combined enzyme activities in the two subfractions was reduced to about half that in the total microsomal fraction of the gland before the subfractionation.

Distribution of cytochrome P-450 between the microsomal subfractions of porcine and rat adrenal

The cytochrome P-450 content in the subcellular preparations obtained by differential centrifugation of the adrenal homogenates was measured by the relative

TABLE II

DISTRIBUTION OF THE MICROSOMAL ENZYMES BETWEEN THE MICROSOMAL SUBFRACTIONS OF PORCINE AND RAT ADRENALS

[4-^{14}C]Pregnenolone (1.58 μmoles, $13 \cdot 10^4$ counts/min) for the assay of the Δ^5-3β-hydroxysteroid dehydrogenase, and [4-^{14}C]progesterone (1.59 μmoles, $10 \cdot 10^4$ counts/min) for the assay of the 17α- and 21-hydroxylases were incubated with 2.0 ml of the submicrosomal suspension which had been used to determine the RNA concentration of porcine adrenal (see legend of Table I), at 37° for 60 min under a gas phase of O_2–CO_2 (95:5, v/v). The final volume of the incubation mixture and final concentration of cofactor were 5 ml per flask and 480 μM, respectively. The adrenal microsomal fraction obtained from 60 rats was suspended in 18.0 ml of 0.33 M sucrose solution, which contained 0.05 M Tris buffer (pH 7.4) and 0.005 M $MgCl_2$. The total microsomal fraction and the smooth- and rough-surfaced subfractions were suspended respectively in 20, 15 and 15 ml of 0.33 M sucrose solution. [4-^{14}C]Pregnenolone (0.32 μmole, $13 \cdot 10^4$ counts/min) in the presence of NAD^+, and [4-^{14}C]progesterone (0.32 μmole, $6 \cdot 10^4$ counts/min) in the presence of NADPH were incubated with 5 ml of the total microsomal fraction (4 mg of protein), 2.5 ml of the smooth-surfaced subfraction (0.35 mg of protein) and 2.5 ml of the rough-surfaced subfraction (0.18 mg of protein). The final volume of the incubation mixture and the concentration of cofactor were 15 ml per flask and 160 μM, respectively. The incubation was carried out for 60 min at 37° under a gas phase of O_2–CO_2 (95:5, v/v) with continuous shaking.

Enzyme	*Fraction**	*Enzyme activity*			
		mμmoles products per mg protein		*mμmoles products per g adrenal*	
		Pig	*Rat*	*Pig*	*Rat*
Δ^5-3β-Hydroxysteroid dehydrogenase	t-Microsomes	73	67	333	2308
	s-Microsomes	159	635	145	1937
	r-Microsomes	30	16	5	28
	Ratio**	5.3	39.7	29.0	69.2
21-Hydroxylase	t-Microsomes	49	62	214	2170
	s-Microsomes	86	401	78	1217
	r-Microsomes	3	4	0.3	5
	Ratio	29.7	100.2	26.0	243.4
17α-Hydroxylase	t-Microsomes	26	—	113	—
	s-Microsomes	49	—	45	—
	r-Microsomes	5	—	1	—
	Ratio	9.8	—	45.0	—

* t-, s- and r-microsomes, respectively, denote the microsomal fraction, which was reserved before subfractionation by density-gradient centrifugation, and smooth- and rough-surfaced subfractions of the adrenal microsomal fraction.

** Smooth-surfaced subfraction/rough-surfaced subfraction.

intensity at 450 mμ in the CO-difference spectra, as shown in Table III.Cytochrome P-450 was concentrated in the mitochondrial and microsomal fractions of the adrenal glands, but not in the supernatant fluid obtained by centrifugation at 105 000 $\times g$. After subfractionation of the adrenal microsomal fraction by density-gradient centrifugation, the cytochrome P-450 of the microsomal fraction was located mainly in the smooth-surfaced subfraction which contained no ribosomes, and the content of cytochrome P-450 in the smooth-surfaced subfraction was increased above that in the total microsomal fraction. A difference spectrum of the microsomal smooth-surfaced subfraction obtained from porcine adrenal glands is shown in Fig. 2, which illustrates the presence of the complex of CO-reduced cytochrome P-450 by its characteristic absorption at 450 mμ.

TABLE III

SUBCELLULAR DISTRIBUTION OF CYTOCHROME P-450 IN PORCINE AND RAT ADRENAL GLANDS

Each of the subcellular fractions was divided into two cuvettes into which a few crystals of sodium dithionite were evenly distributed. The contents of one cuvette were then bubbled with CO for 2 min, and the difference spectra of the CO complex of the reduced cytochrome P-450 *minus* the spectral contribution of the reduced pigments, were recorded from wavelengths of 340 mμ to 700 mμ by a recording spectrophotometer with a diffuse reflectance attachment (Hitachi–Perkin–Elmer, EPS-3T).

Subcellular fraction	*Cytochrome P-450 content* ($\Delta A_{450-500}$ *mμ/cm*)	*Protein (mg/ml)*	*Specific activity of cytochrome P-450 (ΔA per mg protein/ml)*
Porcine adrenal			
Mitochondrial fraction	0.370	2.1	0.176
Total microsomal fraction	0.295	3.7	0.079
Smooth-surfaced subfraction	0.305	1.6	0.191
Rough-surfaced subfraction	0.000	0.3	0.000
Supernatant fluid from centrifugation at 105 000 × *g*	0.000	8.8	0.000
Rat adrenal			
Mitochondrial fraction	0.425	4.4	0.097
Total microsomal fraction	0.125	3.2	0.039
Smooth-surfaced subfraction	0.035	0.4	0.088
Rough-surfaced subfraction	0.000	0.1	0.000
Supernatant fluid from centrifugation at 105 000 × *g*	0.000	6.6	0.000

Effect of CO on the activity of 21-hydroxylase in the rat adrenal smooth-surfaced subfraction

When progesterone was incubated under CO–O_2 (95:5, v/v) or Ar–O_2 (95:5, v/v) for 5 min with the smooth-surfaced subfraction which had been bubbled with O_2, inhibition of 21-hydroxylase activity by CO was demonstrated, as shown in Table IV.

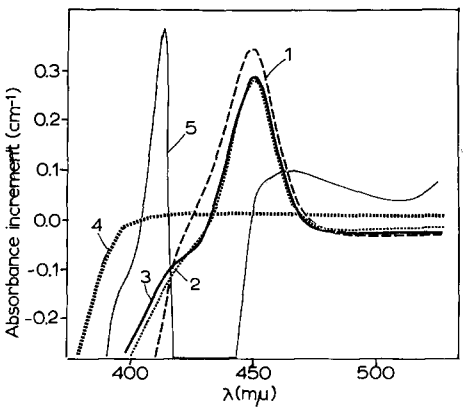

Fig. 2. The difference spectra of cytochrome P-450 in the subcellular fractions. The suspension of porcine mitochondrial fraction (2.1 mg protein/ml), total microsomal fraction (3.7 mg protein/ml), smooth-surfaced subfraction (1.6 mg protein/ml), rough-surfaced subfraction (0.3 mg protein/ml) and the supernatant fluid after centrifugation at 105 000 × *g* (8.8 mg protein/ml), were examined by difference-spectrometric analysis as mentioned in the legend of Table III. Curve 1, mitochondrial fraction; Curve 2, total microsomal fraction; Curve 3, smooth-surfaced subfraction; Curve 4, rough-surfaced subfraction; Curve 5, supernatant fluid from centrifugation at 105 000 × *g*.

TABLE IV

EFFECT OF CO ON 21-HYDROXYLASE ACTIVITY IN THE MICROSOMAL SMOOTH-SURFACED SUBFRACTION OF RAT ADRENALS

The smooth-surfaced subfraction obtained from the adrenal homogenates of 60 rats, was suspended in 13.0 ml of 0.33 M sucrose solution, and the suspension was divided equally into three test tubes (4 ml each), into which 7.0 ml of 0.33 M sucrose solution containing 2 mg NADPH and 0.2 ml propylene glycol had been added, in the dark with cooling. Then, the submicrosomal suspensions (5 ml, 1.72 mg protein per flask) which had been bubbled with O_2, Ar and CO, were introduced into the flasks, to which [4-^{14}C]progesterone (0.1 μmole, $9.6 \cdot 10^4$ counts/min) in propylene glycol had been added, and the air phase had been replaced with the indicated gas. The flasks were then incubated in the dark at 37° for 5 min.

Gas previously bubbled	*Atmosphere (95:5, v/v)*	*Product (11-deoxycorticosterone) (μg)*	*21-Hydroxylase*	
			mμmoles product per mg protein	*Inhibition (%)*
O_2	Ar–O_2	6.09	11.3	0
O_2	CO–O_2	2.40	4.5	60
Ar	Ar–O_2	5.25	9.7	14
Ar	CO–O_2	1.38	2.5	78
CO	Ar–O_2	3.90	7.2	36
CO	CO–O_2	1.35	2.5	78

Under a CO-free atmosphere, the smooth-surfaced subfraction which had been bubbled with Ar shortly before the incubation produced less 11-deoxycorticosterone than the subfraction bubbled with O_2, though this was greater than the amount produced by the suspension which had been bubbled with CO. It was suggested that the O_2 dissolved in the medium was increased by bubbling through O_2 but reduced by bubbling through Ar or CO. Furthermore, by bubbling CO into the incubation mixture, the inactive complex of cytochrome P-450 reduced by NADPH with CO was formed but the reduced cytochrome P-450 was released more rapidly in the atmosphere of O_2–Ar (5:95, v/v) than in O_2–CO (5:95, v/v).

Determination of K_m for the substrates

In accordance with routine kinetic analysis, a fixed amount of enzyme preparation (2.2 mg of protein) was incubated for 20 min with various amounts of the substrate (0.02–0.51 μmole), the amounts of the products were then analyzed after incubation.

The reciprocal of substrate concentration was plotted against the reciprocal of the amount of metabolite obtained. The Michaelis constant for the substrate was then estimated graphically.

The K_m value of the adrenal Δ^5-3β-hydroxysteroid dehydrogenase for pregnenolone was 0.2 mM. On the other hand, the K_m of 17α-hydroxylase for progesterone was 0.13 mM. The K_m of adrenal 21-hydroxylase was evaluated as 99 μM for progesterone, and as 0.22 mM for 17α-hydroxyprogesterone.

Time-course studies of the enzymes in the smooth-surfaced submicrosomal fraction

The activities of the 17α- and 21-hydroxylases reached a plateau after 40 min of incubation, while Δ^5-3β-hydroxysteroid dehydrogenase with Δ^5–Δ^4 isomerase activity increased continuously with an incubation time of up to 180 min.

When progesterone was incubated with the smooth-surfaced microsomal fraction, the substrate decreased exponentially with incubation time, while 11-deoxycorticosterone and 11-deoxycortisol gradually increased (Fig. 3). 17α-Hydroxyprogesterone transiently increased, reaching its maximum at about 60 min after incubation and then gradually decreased, indicating that progesterone was the substrate for both 17α- and 21-hydroxylases, and that 17α-hydroxyprogesterone is the main intermediate between progesterone and 11-deoxycortisol, although 11-deoxycortisol was produced from 11-deoxycorticosterone to a limited extent.

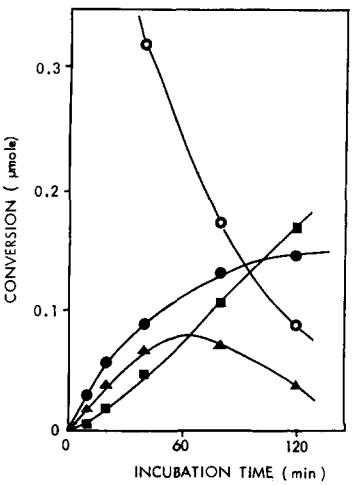

Fig. 3. Dynamic studies on the production of 11-deoxycorticosterone, 17α-hydroxyprogesterone, and 11-deoxycortisol from [4-^{14}C]progesterone by porcine adrenal smooth-surfaced subfraction. Subfractionation of the adrenal microsomal fraction was carried out by the same procedure as mentioned in Table V. After [4-^{14}C]progesterone (0.64 μmole, $4 \cdot 10^4$ counts/min) was incubated in the presence of 1 mg NADPH as cofactor, the amounts of progesterone (○—○), 11-deoxycorticosterone (●—●), 17α-hydroxyprogesterone (▲—▲) and 11-deoxycortisol (■—■) isolated from the incubation flask, were plotted against the time of incubation.

Relationship between the enzyme concentration and the amount of the products

It was confirmed that the activities of the smooth-surfaced microsomal enzymes related to corticoidogenesis of porcine adrenals were proportional to enzyme concentration. A linear relationship was noted between the enzyme preparation and the amount of products converted by the 17α- and 21-hydroxylases over the range 0.18–1.5 mg of protein per flask. In the case of Δ^5-3β-hydroxysteroid dehydrogenase, the linearity of this relationship was observed in the range 0.20–0.72 mg of protein in the preparation.

Substrate specificity of the enzymes related to steroidogenesis

As shown in Table V, the order of substrate preference of the Δ^5-3β-hydroxysteroid dehydrogenase was: pregnenolone > 17α-hydroxypregnenolone > dehydroepiandrosterone ≫ cholesterol in the case of the procine adrenal enzymes, but in the case of rat adrenal, dehydroepiandrosterone was oxidized faster than pregnenolone or 17α-hydroxypregnenolone, by the dehydrogenase in the smooth-surfaced micro-

TABLE V

SUBSTRATE SPECIFICITIES OF THE ENZYMES IN THE MICROSOMAL SMOOTH-SURFACED SUBFRACTION OF PORCINE AND RAT ADRENALS

The smooth-surfaced subfraction obtained from the homogenates of porcine adrenal (55 g wet wt.) was suspended in 55 ml of 0.33 M sucrose solution which contained 0.05 M Tris buffer (pH 7.4) and 0.005 M $MgCl_2$. [4-^{14}C]Cholesterol (1.29 μmoles, $10 \cdot 10^4$ counts/min), [4-^{14}C]pregnenolone (1.58 μmoles, $10 \cdot 10^4$ counts/min), 17α-[7α-^{3}H]hydroxypregnenolone (1.50 μmoles, $81 \cdot 10^4$ counts/min) and [4-^{14}C]dehydroepiandrosterone (1.74 μmoles, $9 \cdot 10^4$ counts/min) were incubated with 3 ml of the suspension of the smooth-surfaced subfraction in the presence of 2 mg NAD^+ for 1 h at 37°. 17α-[4-^{14}C]hydroxyprogesterone (0.61 μmole, $7 \cdot 10^4$ counts/min) for the 21-hydroxylase, 11-[4-^{14}C]deoxycorticosterone (0.61 μmole, $10 \cdot 10^4$ counts/min) for the 17α-hydroxylase, and [4-^{14}C]pregnenolone (0.63 μmole, $10 \cdot 10^4$ counts/min) and [4-^{14}C]progesterone (0.64 μmole, $13 \cdot 10^4$ counts/min) for the above two hydroxylases were examined as substrates. The substrates were incubated with 1.3 ml suspension of the smooth-surfaced subfraction (4.4 mg protein) in the presence of NADPH (2 mg), for 1 h at 37°. The final volume of the incubation mixture was 5 ml. In the case of the rat adrenals, the smooth-surfaced subfraction of adrenals obtained from 45 rats was suspended in 40 ml of 0.33 M sucrose solution which contained 0.05 M Tris buffer (pH 7.4) and 0.005 M $MgCl_2$. [4-^{14}C]Cholesterol (0.26 μmole, $10 \cdot 10^4$ counts/min), [4-^{14}C]pregnenolone (0.32 μmole, $10 \cdot 10^4$ counts/min), 17α-[7α-^{3}H]hydroxypregnenolone (0.30 μmole, $81 \cdot 10^4$ counts/min) and [4-^{14}C]dehydroepiandrosterone (0.35 μmole, $9 \cdot 10^4$ counts/min) were incubated with 2.0 ml of the suspension (0.22 mg protein) in the presence of NAD^+ for 1 h at 37°. [4-^{14}C]Pregnenolone (0.16 μmole, $10 \cdot 10^4$ counts/min), [4-^{14}C]progesterone (0.16 μmole, $6 \cdot 10^4$ counts/min) and 17α-[4-^{14}C]hydroxyprogesterone (0.15 μmole, $12 \cdot 10^4$ counts/min), were incubated with 2.0 ml of the smooth-surfaced subfraction (0.22 mg protein) in the presence of NADPH (2.0 mg), for 1 h at 37°. The final volume of the incubation mixture was 5 ml.

Enzyme	*Substrate*	*Enzyme activity (mμmoles products per mg protein/h)*
Pig		
Δ^5-3β-Hydroxysteroid dehydrogenase	Cholesterol	1
	Pregnenolone	109
	17α-Hydroxypregnenolone	76
	Dehydroepiandrosterone	68
21-Hydroxylase	Pregnenolone	0.0
	Progesterone	55
	17α-Hydroxyprogesterone	55
17α-Hydroxylase	Pregnenolone	3
	Progesterone	43
	11-Deoxycorticosterone	3
Rat		
Δ^5-3β-Hydroxysteroid dehydrogenase	Cholesterol	8
	Pregnenolone	319
	17α-Hydroxypregnenolone	406
	Dehydroepiandrosterone	523
21-Hydroxylase	Pregnenolone	17
	Progesterone	671
	17α-Hydroxyprogesterone	430

somal fraction. When cholesterol was incubated with the smooth-surfaced subfraction of rat and porcine adrenal glands, the product was identified as cholestenone or cholest-4-en-3-one by the method of isotope dilution. Pregnenolone was converted, with a very small yield, to 3β,21-dihydroxypregn-5-en-20-one by the 21-hydroxylase in the smooth-surfaced microsomal fraction of rat adrenals, but this transformation was not observed at all in the case of the porcine adrenal preparation. Progesterone and 17α-hydroxyprogesterone showed a similar affinity for the 21-hydroxylase in the smooth-

surfaced microsomal fraction of porcine adrenals, but the hydroxylase in the rat adrenal smooth-surfaced microsomal fraction showed a lower affinity for non-physiological steroid in rats or 17α-hydroxyprogesterone than for progesterone. Pregnenolone, progesterone and 11-deoxycorticosterone were converted to their respective 17α-hydroxylated derivatives by the 17α-hydroxylase in smooth-surfaced subfraction obtained from porcine adrenals, and among the three substrates, progesterone was found to be the most prefered substrate for the 17α-hydroxylase. It was noted that the small amount of 11-deoxycorticosterone was transformed into 11-deoxycortisol by the microsomal smooth-surfaced subfraction of porcine adrenals.

DISCUSSION

Some of the adrenocortical enzymes related to corticoidogenesis, such as Δ^5-3β-hydroxysteroid dehydrogenase[12], 21-hydroxylase[13] and 17α-hydroxylase[14], have been reported to exist in the microsomal fraction (10 000–105 000 $\times g$ precipitate). By electron-microscopic observation, it was found in the present study that the two subfractions which were obtained by sucrose density-gradient centrifugation of the microsomal fraction at 105 000 $\times g$ for 18 h without CsCl, showed remarkably different features, as shown in Fig. 1. In comparison to our previous results on the enzyme activities in the adrenal submicrosomal fraction of rat which was separated in the presence of CsCl, Cs^+ seems to reduce the enzyme activities examined[7]. Biochemical analysis of adrenal microsomal subfractions indicates a low ratio of RNA to protein in the smooth-surfaced subfraction and a high ratio in the rough-surfaced one. This is in agreement with the results previously reported on the two different types of hepatic microsomal fractions[15–17], the adrenal submicrosomal fractions separated in the presence of CsCl[7], and also with the electron-microscopic observation which reveals the exclusive distribution of ribosomes into the rough-surfaced fraction (Fig. 1). Judging from the protein content of the two subfractions of adrenal microsomes of rat and pig, the smooth-surfaced microsomal fraction constituted the bulk of the microsomal fraction in the adrenals. This finding was supported by the cytological observation that agranular endoplasmic reticula were abundant in adrenocortical cells. The ratio of RNA/protein in the rough-surfaced subfraction was estimated to be about 0.50 and 0.42 for porcine and rat adrenals, respectively, indicating that this subfraction consists of mostly free ribosomes whose RNA/protein ratio is known to be 0.5. This is in agreement with the electron-microscopic observation of this fraction.

The lutein cells of the corpus luteum obtained from hypophysectomized animals, had less developed agranular endoplasmic reticula, but after administration of prolactin to hypophysectomized rats, the lutein cells were found to have abundant and developed agranular endoplasmic reticula[6].

It was reported by HAGERMAN[18] that the specific activity of testicular C_{17}–C_{20} lyase was somewhat enriched in the smooth-surfaced fraction (which sedimented in the presence of Mg^{2+} but not Cs^+), even though significant activity of the enzyme was also detected in the rough-surfaced subfraction. Further studies on the submicrosomal distribution of testicular enzyme activities and of cytochrome P-450 related to androgen production are to be published shortly elsewhere[19].

In the present experiment, the activities of the microsomal enzymes related to corticoidogenesis were clearly demonstrated to be localized in the smooth-surfaced

microsomal fraction which was lacking in ribosomes, but not in the other subfraction. The results of subcellular distribution of cytochrome P-450 in porcine and rat adrenals in this paper are in agreement with the results reported by HARDING *et al.*[20]. It was reported that the cytochrome P-450 in the hepatic microsomal fraction of rabbit[3] and, as mentioned in this paper, in the adrenal microsomal fraction of pig and rat was concentrated exclusively in the smooth-surfaced subfraction. Among the microsomal enzyme activities of adrenals, the 21-hydroxylating system consists of the flavoprotein, its NADPH-linked reductase, non-heme iron protein and cytochrome P-450, *etc.*[21], and probably the same would be applicable for the 17α-hydroxylase, while the Δ^5-3β-hydroxysteroid dehydrogenase and the Δ^5–Δ^4 isomerase did not involve cytochrome P-450. In this connection, the submicrosomal distribution of cytochrome P-450 was consistent with the distribution of 17α- and 21-hydroxylase activities between the adrenal submicrosomal fractions. It is therefore suggested that the cytochrome P-450 in the smooth-surfaced subfraction is involved as an essential component of the 21- and 17α-hydroxylase systems as the direct site of O_2 activation, similar to the role of mitochondrial cytochrome P-450 in the course of 11β-hydroxylation[22,23] and cleavage of cholesterol side-chain[24]. Also, 21-hydroxylation by the adrenal enzyme preparation was inhibited by CO, but was reactivated by light[13,25]. Recently, it was reported, however, that the presence of cytochrome P-450 was not confirmed in the purified preparation of the 21-hydroxylase obtained from sheep adrenal glands[26]. In the present study, however, involvement of the cytochrome P-450 in adrenal 21-hydroxylation was confirmed by the inhibition of the enzyme by CO in the adrenal smooth-surfaced subfraction.

After subfractionation of the adrenal microsomal fraction, the total enzyme activity related to hydroxylation in the subfractions was remarkably reduced, probably due to solubilization and/or inactivation of one or more components of the hydroxylase systems by the hypertonic sucrose solution used. In the case of the testicular submicrosomal fraction, addition of a heat-stable component in the supernatant fluid after centrifugation at 105 000 $\times g$, reactivated the 17α-hydroxylase and C_{17}–C_{20} lyase activities in the smooth-surfaced microsomes which had been treated with the hypertonic sucrose solution[19].

An interesting finding in relation to the electron-transporting enzymes was that the activities of the NADPH-oxidase[2] and NADPH-diaphorase[16] were also found in the smooth-surfaced microsomal fraction of liver. In this connection, it should be noted that 17α-hydroxylase[27] and 21-hydroxylase[13] required NADPH as a cofactor. On the other hand, glucose-6-phosphatase was present almost exclusively in the rough-surfaced subfraction of the hepatic microsomal fraction[16].

Substrate specificities of the Δ^5-3β-hydroxysteroid dehydrogenase[28] and 21-hydroxylase[13] of bovine adrenal glands have been reported, and in this paper, species specificity between pig and rat was detected in the substrate specificity of Δ^5-3β-hydroxysteroid dehydrogenase. Pregnenolone was most rapidly dehydrogenated by the porcine adrenal enzyme, while the dehydrogenase of rat adrenal preferentially converted dehydroepiandrosterone to androstenedione. When 11-deoxycorticosterone was incubated with the smooth-surfaced microsomal fraction obtained from porcine adrenals, production of 11-deoxycortisol was unexpectedly observed though the yield was very small. The 17α-hydroxylase in normal testicular and adrenal glands had specific preference for progesterone as the substrate, but it was reported that the

enzyme in interstitial cell tumor participated in the catalysis of 17α-hydroxylation of 21-hydroxylated steroids[29,30]. In the present studies, conversion of pregnenolone to 3β,21-dihydroxypregn-5-en-20-one by 21-hydroxylase in rat adrenal glands was established. Incubation of 3β,21-dihydroxypregn-5-en-20-one with the homogenates of bovine adrenal glands resulted in the production of 11-deoxycorticosterone[31], and this result suggests that the Δ^5-3β-hydroxysteroid dehydrogenase system is also operative upon even 21-hydroxylated pregnenolone.

In the routine assay of the enzymes, the K_m values of the 17α- and 21-hydroxylases for progesterone, calculated by the graphical method, would be somewhat underestimated because the two microsomal hydroxylases of porcine adrenals are competitively sharing the same substrate. On the other hand, the concentration of the substrate steroids would be less than the amount required to saturate the enzyme because of their low solubility in aqueous solution. Nevertheless, formation of the product increased linearly with the amount of enzyme added except for the Δ^5-3β-hydroxysteroid dehydrogenase.

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