Bioenergetics (1976), 8, 55–71

Steroidogenesis in the Zona Glomerulosa of the Adrenal Cortex II. Distribution of Cytochrome P-450 in the Zona Glomerulosa of the Bovine Adrenal Cortex

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Received 10 November 1975

Abstract

Microsomes were obtained from the zona glomerulosa of the bovine adrenal cortex. Contamination of microsomes with other cellular organelles was examined using various marker enzymes and the electron microscope. Distribution of cytochrome P-450 in the zona glomerulosa was studied using various fractions including microsomes, described above, and mitochondria. The amount of cytochrome P-450 in mitochondria and in microsomes was determined to be 0.73 and 0.32 nmol/mg protein, respectively. The CO difference spectrum was affected not only by the concentration of added deoxycholate but also by the incubation time after addition. Approximately 40–50% of cytochrome P-450 in the samples was converted to cytochrome P-420 within 20–30 sec of incubation with deoxycholate.

The content of RNA, phospholipids, and cytochrome b_5 in microsomes obtained from the zona glomerulosa is also evaluated in comparison to that in microsomes obtained from the zona fasciculo-reticularis.

Introduction

Steroidogenesis in the adrenal cortex has been studied extensively by many workers. Mitochondria of adrenocortical tissue possess an electron transport system, distinct from the classical respiratory chain, that is concerned with the steroid hydroxylation reaction [1–6] most notably the 11β-hydroxylation of deoxycorticosterone [7–8]. Furthermore, cytochrome P-450 has been shown to be present in adrenal cortex mitochondria [1, 8] and has been implicated in the 11βhydroxylation reaction [9–12] as well as in the conversion of cholesterol to pregnenolone [3, 13]. Adrenal cortex microsomes also possess cytochrome P-450 which is concerned with 21-hydroxylation [14]. All these data described above, however, are restricted to the whole cortex or to the zona fasciculata (+ the zona reticularis). Up to now, there are no available data except one [15] concerning the zona glomerulosa, dealing with the mixed function oxidase systems for the hydroxylation of steroids at the level of subcellular fractions.

Ichikawa et al. [15] have reported the concentration of hemoprotein P-450 in the different zones of the adrenal cortex (including the zona glomerulosa) using mitochondria or microsomes isolated from the cow or pig. However, they started with frozen materials and contamination of mitochondria or microsomes of the zona glomerulosa with those of the zona fasciculata has not been examined in their report.

Previously we have reported the isolation procedures for mitochondria from the zona glomerulosa using the bovine adrenal cortex [16] and we have also examined the purity of mitochondria using several marker enzymes [17].

Thus, the purpose of the present communication is to study the distribution of cytochrome P-450 in subcellular fractions obtained from the zona glomerulosa of the adrenal cortex as the first step in the exploration of steroidogenesis in the above-described zone.

Experimental Procedures

Preparation of Adrenal Cortex Mitochondria and Microsomes from the Zona Glomerulosa

Figure 1 is a flow diagram illustrating the fractionation procedure described below. Fresh bovine adrenals were obtained at a local slaughter house, chilled in ice immediately, and brought to the laboratory within 1–1.5 hr of the death of the animal. The zona glomerulosa was scraped and collected according to the method reported previously [16, 17]. The isolation medium employed in the present study contained 0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, and 0.1 mM EDTA. The zona

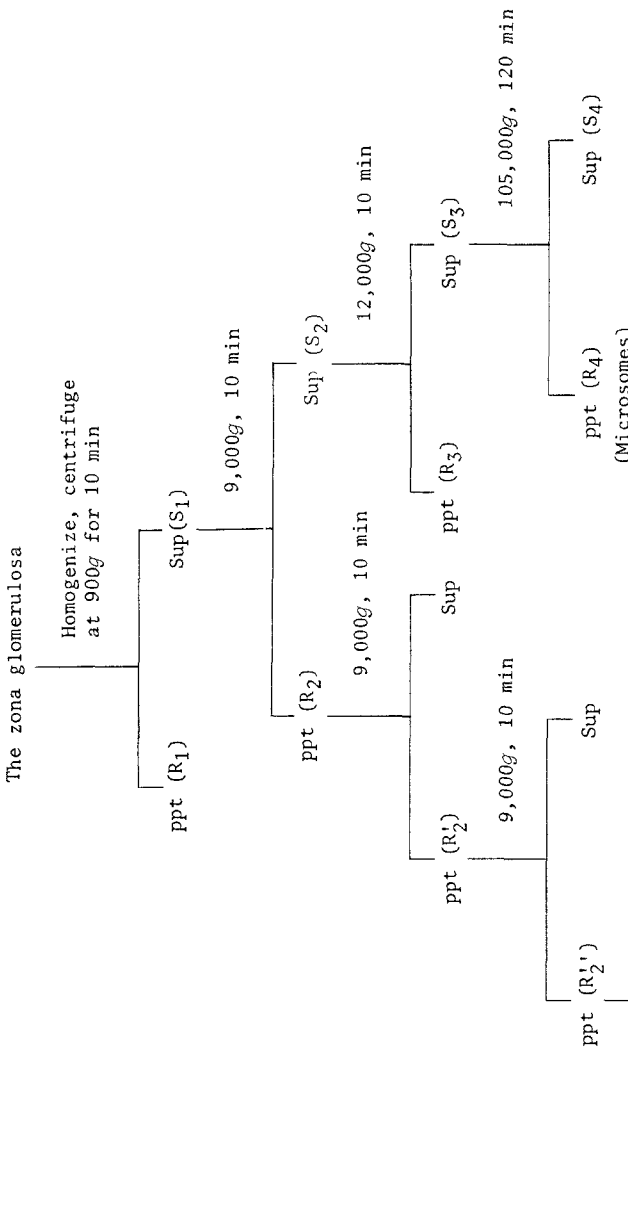


Figure 1. Flow diagram for the preparation of mitochondria and microsomes from the zona glomerulosa of the bovine adrenal cortex.

fasciculoreticularis was also collected. After obtaining a crude mitochondrial fraction (R_2), the supernatant fluid (S_2) was first centrifuged at 12,000 *g* for 10 min. The supernatant fluid thus obtained (S_3) was then centrifuged for 120 min at 105,000 *g* in a Spinco model L ultracentrifuge to obtain the microsomal fraction. The yield of the microsomal fraction to the zone glomerulosa is shown in Table I. The

TABLE I. Yield of the microsomal fraction obtained from the zona glomerulosa[a]

	Zona glamerulosa	Zona fasciculo reticularis
R_4/one gland (mg)	16.26 ± 2.42	61.38 ± 8.04
R_4/scraped tissues (%)	1.55 ± 0.17	1.18 ± 0.09
R_4/homogenates (%)	6.41 ± 1.29	6.80 ± 1.23

[a] Data are average of 10 different experiments (mean ± SD) Each experiment consisted of 15-20 adrenal glands.

whole procedure was carried out at 4° C. The mitochondrial and the microsomal fractions from the zona fasciculoreticularis were obtained with the same fractionation procedures as were used for the fractions from the zona glomerulosa. Purity of the fractions was evaluated by enzymic tests using various reference enzymes [17] and also by electron microscopy. Subfractions were also obtained from the mouse liver by the same method as described for the zona glomerulosa.

Analytical Procedures

The absolute and difference absorption spectra of various subcellular fractions were measured with a Shimazu multipurpose recording spectrophotometer model MPS-50L. The contents of cytochrome P-450 and P-420 were determined from the CO difference spectrum of dithionite-reduced samples, assuming a value of 91 $mM^{-1}\,cm^{-1}$ for the molar extinction increment between 450 and 490 nm for P-450, and a value of 110 $mM^{-1}\,cm^{-1}$ between 420 and 490 nm for P-420 [18]. Cytochrome b_5 was determined from the difference spectrum between NADH-reduced and air-saturated microsomal fractions. The increment of molar extinction between 409 and 429 nm in the difference spectrum was assumed to be 185 $mM^{-1}\,cm^{-1}$ [18]. The contamination of hemoglobin was tested spectrophotometrically in the presence and absence of CO in the test tube.

Content of RNA

The microsomal fraction obtained from the zona glomerulosa was fractionated by the Smith–Thanhauser–Schneider method [19] as

modified by Mizuno and Whiteley [20]. Each fraction was assayed for RNA (absorbancy at 260 nm after 18 hr of hydrolysis in 0.3 N KOH at 37° C).

Estimation of Phospholipids

Content of phospholipids in a microsomal fraction was determined by the method of Hoeflmayr et al. [21] as modified by Kushiro and Fukui [22]. Inorganic phosphate released was measured according to the method of Fiske and Subbarow [23].

Protein was determined by the biuret method of Gornall et al. [24] using bovine serum albumin as standard. Increase in absorption in the biuret reaction due to heme in the test samples was avoided by using the reference containing the hemoprotein in 4% NAOH [15].

Electron Microscopy

Procedures for electron microscopy are described in full in the previous communication [16]. A 1% solution of phosphotungstic acid or 2% solution of ammonium molybdate (adjusted to pH 7.4) was used for negative staining [17].

Results

Purity of the Microsomal Fraction Obtained from the Zona Glomerulosa

The purity of the microsomal fraction obtained from the zona glomerulosa was examined using several marker enzymes (Table II). In the present study the supernatant S_2 fraction was first centrifuged for 10 min at 12,000 *g* as described in the previous section. The microsomal fraction was then prepared from the supernatant fraction (S_3) thus obtained.

As shown in Table II, the R_3 fraction obtained from the zona glomerulosa is significantly contaminated with mitochondria as measured by cytochrome oxidase activity, compared with the R_3 fraction of the liver. Thus, it seems essential to use the S_3 fraction for the preparation of a microsomal fraction in the case of the zona glomerulosa of the adrenal cortex (electron micrograph of the R_3 fraction is presented later). Both glucose-6-phosphatase and catalase activities in the R_4 fraction of the zona glomerulosa are extremely low compared with those in the liver. Moreover, nearly 50% of the total glucose-6-phosphatase activity was recovered in the S_4 fraction, and more than 60% of the total catalase activity was recovered in that fraction.

TABLE II. Purity of the microsomal fraction obtained from the zona glomerulosa[a,b]

	Glucose-6-phosphatase			Acid phosphatase		
	Z.gl.	Z.fas.-ret.	Liver	Z.gl.	Z.fas.-ret.	Liver
	S.A(T.A)	S.A(T.A)	S.A(T.A)	S.A(T.A)	S.A(T.A)	S.A(T.A)
Homogenates	10.8(100.0)	8.3(100.0)	37.0(100.0)	18.2(100.0)	13.8(100.0)	8.4(100.0)
R_3	6.0(3.1)	9.9(5.2)	57.7(5.1)	15.7(4.3)	18.8(6.0)	16.2(4.9)
S_3	21.6(60.3)	21.9(70.3)	44.1(61.1)	28.6(47.0)	22.9(44.2)	13.2(45.2)
R_4	8.0(6.5)	7.9(4.5)	140.5(52.6)	21.3(8.5)	20.9(7.9)	7.1(8.3)
S_4	23.8(46.9)	19.3(49.3)	10.7(7.5)	29.0(34.0)	21.0(32.4)	15.6(36.4)

	Catalase			Cytochrome oxidase		
	Z.gl.	Z.fas.-ret.	Liver	Z.gl.	Z.fas.-ret.	Liver
	S.A(T.A)	S.A(T.A)	S.A(T.A)	S.A(T.A)	S.A(T.A)	S.A(T.A)
Homogenates	4.7(100.0)	4.4(100.0)	179.1(100.0)	65.4(100.0)	65.2(100.0)	92.7(100.0)
R_3	1.0(2.8)	3.0(2.3)	265.3(18.6)	155.4(5.2)	168.8(7.2)	106.3(4.2)
S_3	5.8(68.0)	12.9(66.8)	53.1(16.8)	54.6(4.0)	39.2(2.8)	64.5(3.2)
R_4	1.1(4.8)	2.8(2.9)	66.5(4.4)	82.1(2.9)	65.8(1.9)	50.2(2.7)
S_4	5.8(62.3)	15.3(62.2)	108.5(11.4)	0 (0.0)	0 (0.0)	0 (0.0)

[a] Specific activities (S.A) are expressed in nmol P_i/mg/min for glucose-6-phosphatase and acid phosphatase, n-atoms oxygen/mg/min for cytochoome oxidase and $-\Delta A_{410} \times 10^2$/mg/min for catalase.

[b] Total activity (T.A.) of homogenates is expressed as 100%.

Spectral Studies of Cytochrome P-450 in the Zona Glomerulosa

Spectral studies of reduced and oxidized cytochrome P-450 in the adrenal cortex have been reported by several workers. In the present study an attempt has been made to estimate the amount of cytochrome P-450 in various fractions obtained from the zona glomerulosa. In Fig. 2 a CO difference spectrum of the mitochondrial fraction (three times washed, G-R_2''') is recorded in comparison with that of the mitochondrial fraction obtained from the zona fasciculoreticularis (F-R_2'''). No significant contamination with hemoglobin was detected in the mitochondrial fraction isolated either from the zona glomerulosa or from the zona fasciculoreticularis.

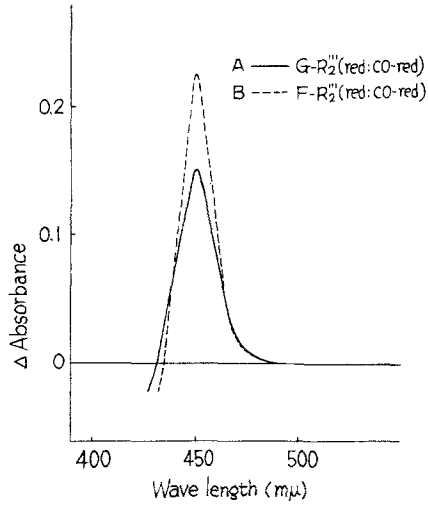


Figure 2. Carbon monoxide difference spectrum of adrenal cortex mitochondria isolated from the zona glomerulosa. Mitochondria of the zona glomerulosa (G-R_2''') were diluted to 6 ml with a 0.1 M Tris-Cl buffer, pH 8.0, and divided equally into two cuvettes. After recording a baseline, the sample cuvette was gassed with CO for 1 min. The sample and reference cuvettes were then reduced with a small amount of dithionite and the difference between CO-reduced and reduced was recorded. Curve B corresponds to curve A except that mitochondria were obtained from the zona fasciculoreticularis.

Contamination of mitochondria isolated from the zona glomerulosa with those isolated from the zona fasciculoreticularis has been eliminated [16]. We have also examined the purity of mitochondria of the zona glomerulosa using several marker enzymes [17]. Thus, the effect of washing on the content of mitochondrial cytochrome P-450 was examined in the present study. As is seen in Table III, the amount of

cytochrome P-450 in washed mitochondria ($G\text{-}R_2'$, $G\text{-}R_2''$, and $G\text{-}R_2'''$) is considerably higher than that in a crude mitochondrial fraction ($G\text{-}R_2$). The amount of cytochrome P-450 is essentially the same among fractions of washed mitochondria. On the other hand, the amount of cytochrome P-450 remained essentially the same before and after washing of mitochondrial fractions obtained from the zona fasciculo-reticularis.

TABLE III. Effect of washing on the contents of cytochrome p-450 in mitochondria isolated from the zona glomerulosa[a]

Fraction	Ex.1	Ex.2	Ex.3	Mean
	(nmol/mg protein)			
$G\text{-}R_2$	0.17	0.12	0.17	0.15
$G\text{-}R_2'$	0.38	0.37	0.37	0.37
$G\text{-}R_2''$	0.39	0.35	0.33	0.36
$G\text{-}R_2'''$	0.35	0.31	0.33	0.33
$F\text{-}R_2$	0.30	0.37	0.23	0.30
$F\text{-}R_2'$	0.32	0.42	0.53	0.42
$F\text{-}R_2''$	0.35	0.41	0.39	0.38
$F\text{-}R_2'''$	0.42	0.40	0.37	0.40

[a] A crude mitochondrial fraction obtained from the zona glomerulosa ($G\text{-}R_2$, see Fig. 1 for explanation) was diluted to 6 ml with a 0.1 M Tris-Cl buffer, pH 8.0, and clarified with deoxycholate at a final concentration of 0.15%. The sample was divided equally into two cuvettes. After recording a base line the content of cytochrome P-450 was measured as described in Fig. 2. The content of cytochrome P-450 in R_2' (washed once), R_2'' (washed two times), and R_2''' (washed three times) was measured in the same way.

In the course of the present study it was found that the content of cytochrome P-450 in a mitochondrial fraction was markedly affected by treatment with deoxycholate; levels were affected by both concentration and duration of exposure to deoxycholate.

The CO difference spectra of deoxycholate-treated mitochondria of the zona glomerulosa are shown in Fig. 3. In this experiment, a sample ($G\text{-}R_2'''$) was diluted with the buffer and divided equally into two cuvettes. After the addition of carbon monoxide to one cuvette, both cuvettes were reduced with a small amount of dithionite. Deoxycholate was added both to the sample and the reference cuvettes at the same time, and the difference spectrum was recorded. As is seen in the figure,

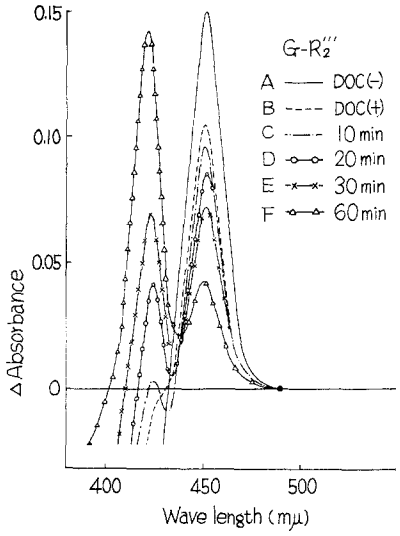


Figure 3. Effect of deoxycholate on carbon monoxide difference spectrum of the adrenal cortex mitochondria isolated from the zona glomerulosa. The sample ($G\text{-}R_2'''$) was diluted to 6 ml with a 0.1 M Tris-Cl pH 8.0, and the sample was divided equally into two cuvettes. Carbon monoxide was added to the sample cuvette, and both the sample and the reference cuvettes were reduced with a small amount of dithionite. Deoxycholate was added to both cuvettes at a final concentration of 0.15%. The final concentration of protein was 2.4 mg/ml.

after incubation of the sample with deoxycholate for 20 sec, formation of P-420 in parallel with disappearance of P-450 is seen already (see curve B). After 20 min, the amount of P-450 decreased to less than a half of the original. This phenomenon is comparable with that in liver microsomes discovered by Omura and Sato [18].

The CO difference spectra of the microsomal fractions obtained from the zona glomerulosa and from the zona fasciculoreticularis are shown in Fig. 4. The difference spectrum shows absorption maxima at 450 and 415 nm. The latter absorption maximum was found to be mainly due to contamination with hemoglobin and to a minor extent due to cytochrome b_5.

Contents of cytochrome P-450 in various fractions of the zona glomerulosa are summarized in Table IV obtained from CO difference spectra using turbid suspensions without deoxycholate treatment. As is shown in the table, the content of cytochrome P-450 in the mitochondrial fraction ($G\text{-}R_2'''$) is about twice as much as that obtained with deoxycholate treatment (cf. Table III and Fig. 3).

Contents of cytochrome P-450 and cytochrome b_5 in mitochondrial

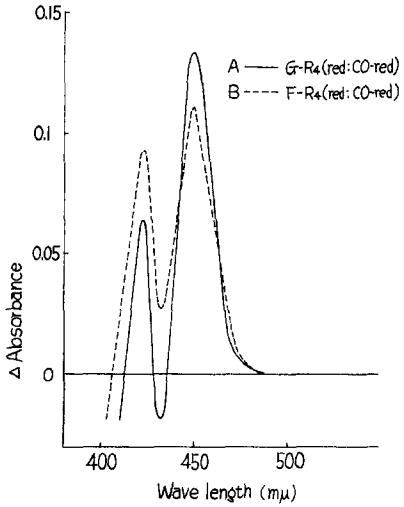


Figure 4. Carbon monoxide difference spectrum of adrenal cortex microsomes isolated from the zona glomerulosa. Experiments were carried out as described in the legend of Fig. 2. The final concentration of protein was 1.5 mg/ml.

TABLE IV. Distribution of cytochrome P-450 in the zona glomerulosa of the adrenal cortex[a]

Fractions	P-450 (nmol/mg protein)
Homogenates	0.14
Crude mitochondria, R_2	0.38
Washed mitochondria, R_2	0.73
Microsomes,	
R_3	0.46
R_4	0.32
S_1	0.29
S_2	0.13
S_3	0.04
S_4	not detectable

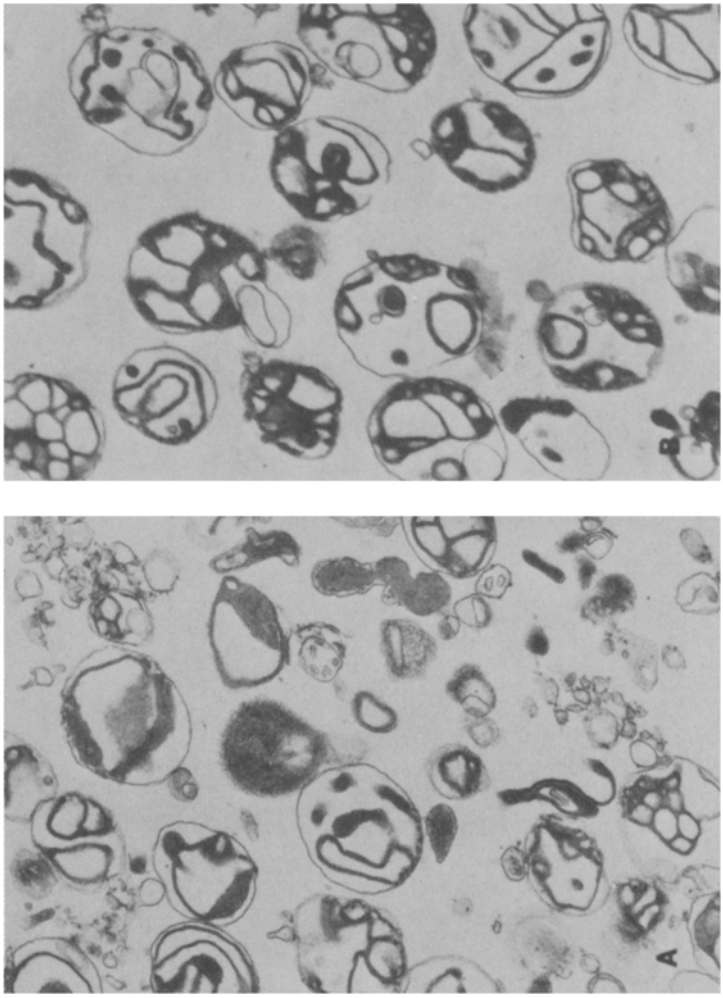
[a] The values were the averages of three different experiments obtained from CO difference spectra of dithionite-reduced samples.

and microsomal fractions from the zona glomerulosa and the zona fasciculoreticularis are summarized in Table V. No distinct difference in cytochrome content was obtained between the two zones specified above.

TABLE V. Contents of cytochrome P-450 and cytochrome b_5 in mitochondrial and microsomal fractions obtained from the zona glomerulosa[a]

Zones	Mitochondria				Microsomes		
	P-450 (nmol/mg protein)		P-450 (nmol/mg protein)		b_5 (nmol/mg protein)	Phospholipids/mg protein	RNA (μg/mg protein)
Zona gl.	0.73	0.57[b]	0.32	0.70[b]	0.06	0.83	82.5
		1.00[b] (Zona fas.)		1.50[b] (Zona fas.)			
Zona fas.-ret.	0.96	0.70[b] (Zona ret.)	0.36	0.80[b] (Zona ret.)	0.05	0.78	53.7
						0.54 (Mouse liver)	142.0 (Mouse liver)

[a] Values show the averages of three determinations of different samples.
[b] Data from Ichikawa et al. [15].



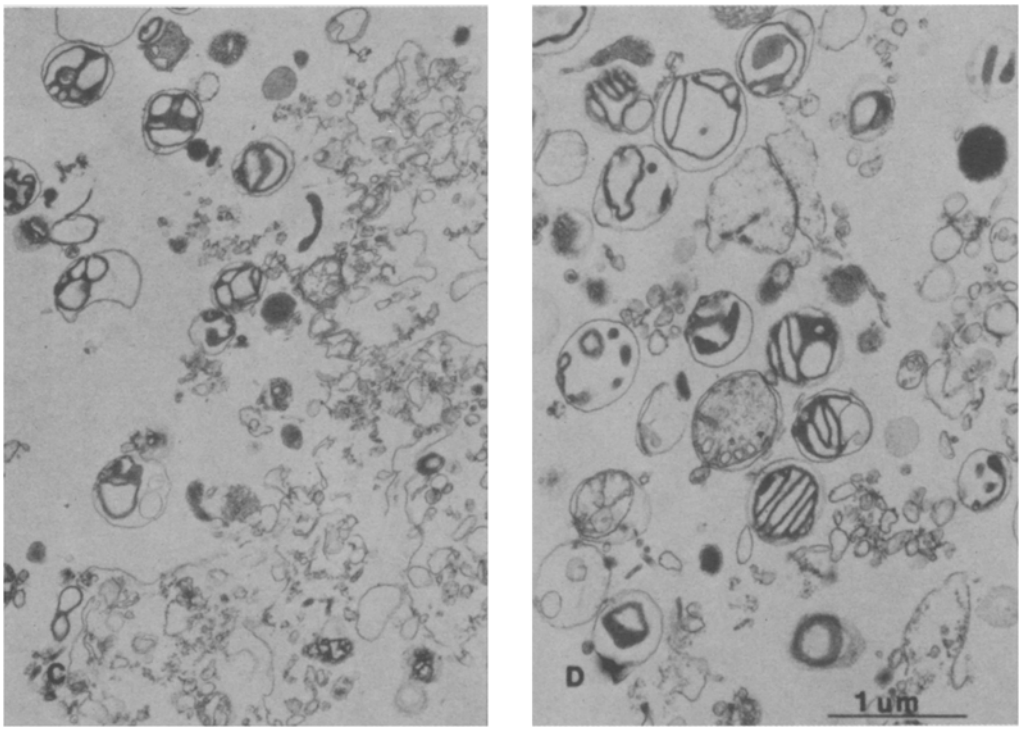


Figure 5. Electron micrographs of subcellular fractions obtained from the zona glomerulosa of the adrenal cortex. A crude mitochondrial fraction (R_2, 5A, x 20,000), three-times washed mitochondrial fraction (R_2''', 5B, x 20,000), S_2 fraction (5C, x 20,000), and R_3 fraction (5D, x 20,000) obtained from the zona glomerulosa were fixed in glutaraldehyde and postfixed with osmium tetroxide. (Illustrations reduced 10% for reproduction).

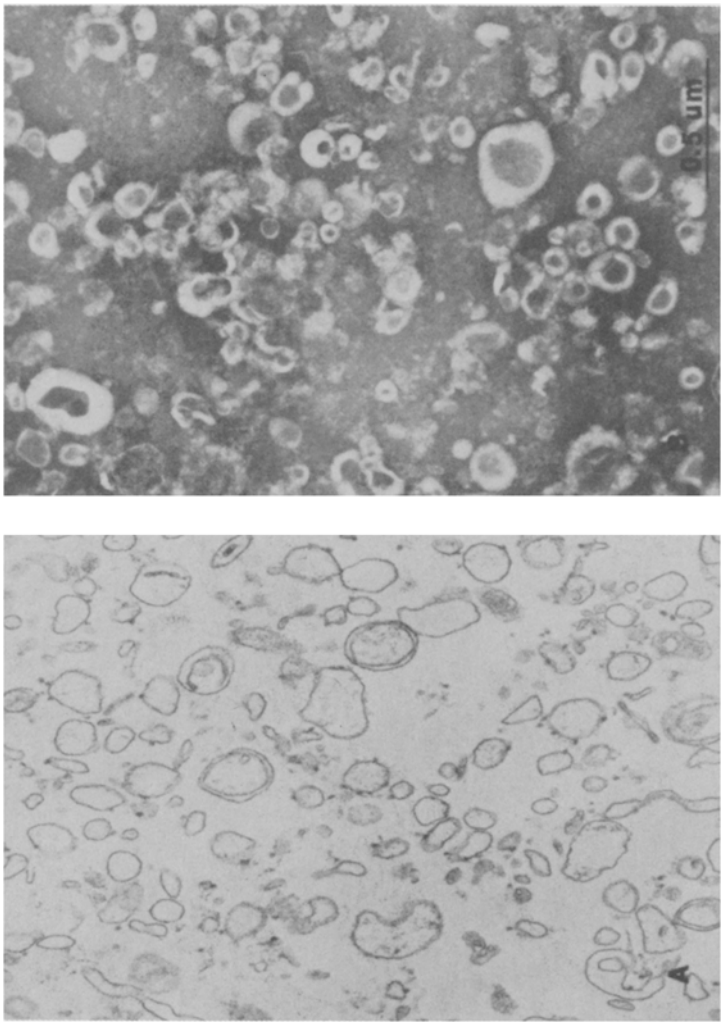


Figure 6. Electron micrographs of microsomal fraction obtained from the zona glomerulosa. (A) Microsomes were fixed in glutaraldehyde, postfixed with osmium tetroxide (×40,000). (B) Negatively stained with 2% ammonium hydroxide (×40,000). (Illustrations reduced 10% for reproduction).

Electron Microscopy

It has been shown in the previous section that a crude mitochondrial fraction obtained from the zona glomerulosa ($G\text{-}R_2$) has a lower amount of cytochrome P-450 than that of the washed mitochondrial fraction ($G\text{-}R_2'''$). When a crude mitochondrial fraction was examined in the electron microscope, it was found to be contaminated with microsomes (Fig. 5A). The contaminants might have resulted in the apparently lower value of cytochrome P-450 in this fraction than that in the washed mitochondrial fraction. After washing the fraction, clean mitochondria were obtained (Fig. 5B). The supernatant S_2 fraction (Fig. 5C), which had been obtained after centrifugation of the S_1 fraction, was composed of microsomes, fragments of membranes (possibly the inner mitochondrial membranes), and small, apparently intact mitochondria. The R_3 fraction was obtained after centrifugation of the S_2 fraction at 12,000 *g* for 10 min (Fig. 5D). This fraction showed fragments of membrane (possibly the inner mitochondrial membrane, since sizes of these fragments are larger than those of microsomes), and small mitochondria and microsomes. Thus, the supernatant S_2 fraction cannot be used as a starting material for the preparation of a microsomal fraction in the case of the zona glomerulosa.

The microsomal fraction obtained from the zona glomerulosa is shown in Fig. 6. No contamination of mitochondria or of mitochondrial fragments is detected morphologically.

Discussion

In the present study distribution of cytochrome P-450 in the zona glomerulosa of the bovine adrenal cortex has been studied. To evaluate steroidogenesis in the zona glomerulosa of the adrenal cortex, it is essential to investigate the distribution of cytochrome P-450, an important component of the mixed function oxidase systems for the hydroxylation of steroids [9, 14, 25–28].

It is well established that mitochondria of the zona fasciculoreticularis contain cytochrome P-450 and that they have vesicular cristae, when revealed in the electron microscope, which are characteristic of mitochondria in steroid-producing organs [29–31]. On the other hand, mitochondria in the zona glomerulosa have tubular or tubulovesicular cristae in situ rather than vesicular cristae. It has also been reported that mitochondria in the zona glomerulosa do not undergo configurational changes, regardless of experimental conditions such as ACTH administration or unilateral adrenectomy or hypophysectomy [32, 33], when examined in the electron microscope.

In the present study it has been shown that mitochondria in the zona

glomerulosa have cytochrome P-450, and thus they might be concerned with steroidogenesis. The data on this point will be published in a separate paper.

Ichikawa et al. have measured the amount of cytochrome P-450 in mitochondria or microsomes isolated from the zona glomerulosa using frozen ox or pig adrenal cortex [15]. They scraped the cortical tissues from the capsule, and then the cortical tissues were cut into thin slices at -20° C. The zona glomerulosa was obtained from the outer portion of the slices using a binocular microscope. When we tested their procedures, it was found that the zona glomerulosa of the bovine or pig adrenal cortex was rather well demarcated from the adjacent zona fasciculata when examined in the light microscope. However, contamination of the zona glomerulosa with the zona fasciculata was apparent when examined in the electron microscope. Thus, in the present study, we have obtained the test sample of the zona glomerulosa from the capsule, leaving the inner portion of the zona glomerulosa attached to the zona fasciculata to avoid contamination. The homogeneity of the mitochondrial fraction isolated from the zona glomerulosa or the zona fasciculoreticularis has been reported in the first communication of this series [17] and has also been confirmed in the present study.

Thus, we believe that the present study is the first to report the distribution of cytochrome P-450 in the zona glomerulosa of the adrenal cortex using uncontaminated materials, although "the zona glomerulosa" used in the present study does not include the whole width of the zone.

It has been reported by Omura and Sato that cytochrome P-450, upon exposure to various agents, is converted to a complex absorbing at 420 nm [18]. The present study also confirms their finding that cytochrome P-450 is strongly affected by the addition of deoxycholate.

Finally it should be stressed again that catalase activity is extremely low in the zona glomerulosa. Kimberg et al. have reported that microbodies in the liver decrease in number when the animal is treated with cortisone [34]. Considering the fact that both the zona glomerulosa and the zona fasciculoreticularis produce steroid hormones, the data presented in this study seem to be meaningful, and we are now studying the role of microbodies in the zona glomerulosa.

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