Common characteristics of the cytochrome P-450 system involved in 18- and 11β-hydroxylation of deoxycorticosterone in rat adrenals

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Abstract 18- and 11β-Hydroxylation of deoxycorticosterone and side chain cleavage of cholesterol were studied in mitochondria and submitochondrial reconstituted systems prepared from rat and bovine adrenals. A mass fragmentographic technique was used that allows determination of hydroxylation of both exogenous and endogenous cholesterol. The following results were obtained.

(1) Treatment of rats with excess potassium chloride in drinking fluid increased mitochondrial cytochrome P-450 as well as 18- and 11β-hydroxylase activity in the adrenals. Cholesterol side chain cleavage was not affected. In the presence of excess adrenodoxin and adrenodoxin reductase, cytochrome P-450 isolated from potassium chloride-treated rats had higher 18- and 11β-hydroxylase activity per nmol than cytochrome P-450 isolated from control rats. The stimulatory effects on 18- and 11β-hydroxylation were of similar magnitude.

(2) Long-term treatment with ACTH increased cholesterol side chain cleavage in the adrenals but had no effect on 18- and 11β-hydroxylase activity. The amount of cytochrome P-450 in the adrenals was not affected by the treatment. It was shown with isolated mitochondrial cytochrome P-450 in the presence of excess adrenodoxin and adrenodoxin reductase that the effect of ACTH was due to increase of side chain cleavage activity per nmol cytochrome P-450. Side chain cleavage of exogenous cholesterol was affected more than that of endogenous cholesterol.

(3) Gel chromatography of soluble cytochrome P-450 prepared from rat and bovine adrenal mitochondria yielded chromatographic fractions having either a high 18- and 11β-hydroxylase activity and a low cholesterol side chain cleavage activity or the reverse. The ratio between 18- and 11β-hydroxylase activity was approximately constant, provided the origin of cytochrome P-450 was the same.

(4) Addition of progesterone to incubations of deoxycorticosterone with soluble or insoluble rat adrenal cytochrome P-450 competitively inhibited 18- and 11β-hydroxylation of deoxycorticosterone to the same degree. Addition of deoxycorticosterone competitively inhibited 11β-hydroxylation of progesterone with the same system. Progesterone was not 18-hydroxylated by the system.

From the results obtained, it is concluded that 18- and 11β-hydroxylation have similar properties and that the binding site for deoxycorticosterone is similar or identical in the two hydroxylations. The possibility that the same specific type of cytochrome P-450 is responsible for both 18- and 11β-hydroxylation of deoxycorticosterone is discussed.

Supplementary key words cholesterol side chain cleavage · mass fragmentography

In a previous work, 18- and 11β-hydroxylation of different steroids in mitochondria and reconstituted systems from rat and bovine adrenals were studied (1). It was found that 18- and 11β-hydroxylase activities with deoxycorticosterone as substrate were unchanged under various assay conditions (suboptimal amounts of substrate, cytochrome P-450, adrenodoxin and/or adrenodoxin reductase). The ratio also remained constant during inhibition with carbon monoxide and metyrapone. From these results, it was suggested that identical or at least very similar types of cytochrome P-450 are involved in 18- and 11β-hydroxylation of deoxycorticosterone. In the present work, we have by different means further investigated the possibility that a single enzyme system is involved in 18- and 11β-hydroxylation of deoxycorticosterone. Treatment of rats with potassium chloride in drinking fluid has been reported to increase 18-hydroxylation of corticosterone (2, 3). It was considered to be of interest to determine whether this increase in 18-hydroxylase activity is followed by a similar increase in 11β-hydroxylase activity. Long-term treatment with ACTH is known to have different effects on different hydroxylations in the adrenals (4). It was also considered to be of interest to study if 11β- and 18-hydroxylation and cholesterol side chain cleavage are affected differently by such treatment.

In some recent work, methods have been described for preparation of fractions of cytochrome P-450 from bovine adrenals that either have a high cholesterol

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side chain cleavage activity and a low 11β-hydroxylase activity or vice versa (5–8). 18-Hydroxylase activity has not been followed through such purifications. The relatively low 18-hydroxylase activity of bovine adrenal cytochrome P-450 make such studies difficult (cf. 1). Rat adrenal cytochrome P-450 has a relatively high ratio between 18-hydroxylase activity and 11β-hydroxylase activity (cf. 1), and in the present work we have therefore tried to subject rat adrenal cytochrome P-450 to partial purification and have studied the ratio between 18- and 11β-hydroxylation of deoxycorticosterone during the procedures.

In previous work from this laboratory (1), it was shown that a crude mitochondrial system as well as a reconstituted cytochrome P-450 system catalyzed both 18- and 11β-hydroxylation of deoxycorticosterone but only 11β-hydroxylation of progesterone. Unlabeled progesterone was found to inhibit both 18- and 11β-hydroxylation of tritium-labeled deoxycorticosterone, and deoxycorticosterone inhibited 11β-hydroxylation of [4-^{14}C]progesterone. In the present work, attempts have been made to determine whether or not these inhibitions are of a competitive nature. Inhibition of a competitive nature would suggest that the same binding site or at least very similar binding sites are involved in 11β-hydroxylation of progesterone and deoxycorticosterone and in 18-hydroxylation of deoxycorticosterone.

MATERIALS AND METHODS

[1,2-$^{3}H_2$]Corticosterone (sp act 40 μCi/μmol) was purchased from New England Nuclear (Boston, MA). [1,2-$^{3}H_2$]Deoxycorticosterone (sp act 10 μCi/μmol), [4-^{14}C]progesterone (sp act 3 μCi/μmol), [4-^{14}C]cholesterol (sp act 59–60 μCi/μmol), and [4-^{14}C]pregnenolone (sp act 59–60 μCi/μmol) were purchased from Radiochemical Centre (Amersham, England). The purity of the labeled compounds was ascertained by means of thin-layer chromatography followed by radio-scanning of the chromatoplates (cf. below). Aldosterone, corticosterone, deoxycorticosterone, and ACTH were obtained from Sigma Chemical Co. (St. Louis, MO). 18-Hydroxycorticosterone was purchased from Steraloids (Pawling, NY), Sephadex G-100 and G-200 were obtained from Pharmacia (Uppsala, Sweden). DEAE–Cellulose was obtained from Whatman (Maidstone, England).

Assay of cytochrome P-450 and NADPH–cytochrome P-450 reductase activity

Cytochrome P-450 was assayed from the absorbance of the carbon monoxide–cytochrome P-450 complex, after reduction with sodium dithionite using an extinction coefficient of 91 cm^{-1} mM^{-1} (9). The concentration of cytochrome P-450 in various preparations is given in **Table 1.** NADPH–Cytochrome P-450 reductase activity was assayed according to Masters, Williams and Kamin (10) and expressed in units (one unit equals reduction of 1 nmol of cytochrome *c* per min). Protein was determined according to Lowry et al. (11).

TABLE 1. Amount of insoluble cytochrome P-450 and cholesterol in submitochondrial preparations from rat adrenals

Preparation	Protein	Cytochrome P-450	Cholesterol
	mg	*nmol*	*μg*
Cytochrome P-450 from ACTH-treated rats	5.4 (5.0–5.7)	5.8 (5.4–6.2)	1.4 (1.2–1.6)
Cytochrome P-450 from corresponding control rats	7.3 (6.8–7.7)	5.9 (5.4–6.1)	3.4 (3.2–3.6)
Cytochrome P-450 from potassium chloride-treated rats	8.8 (8.0–9.7)	12.9 (12.3–13.4)	3.5 (3.4–3.7)
Cytochrome P-450 from corresponding control rats	7.2 (6.7–7.6)	6.4 (6.3–6.4)	1.3 (1.2–1.5)

Total recovery of cytochrome P-450 from the mitochondrial fraction was > 95%. The figures given are the mean and the range of three independent experiments.

Preparation of NADPH–cytochrome P-450 reductase, adrenodoxin and adrenodoxin reductase from bovine adrenals

Bovine NADPH–cytochrome P-450 reductase containing a mixture of adrenodoxin and adrenodoxin reductase was prepared according to Omura et al. (12) and was similar to the preparation used in the previous works (1, 13). The preparation sometimes contained small amounts of soluble cytochrome P-450 showing side chain cleavage activity. This cytochrome P-450 always had very little activity towards deoxycorticosterone (Table 6) which could be neglected under standard assay conditions. If present, the small amount of cholesterol side chain cleavage activity of the NADPH–cytochrome P-450 reductase fractions was subtracted from the total cholesterol side chain cleavage activity in combination experiments (Tables 3 and 5). In some experiments, in which only small amounts of cholesterol side chain cleavage activity were determined, an NADPH–cytochrome P-450 reductase fraction completely free from cytochrome P-450 was used (Table 6). In these cases the crude NADPH–cytochrome P-450 reductase fraction was replaced with a mixture of purified

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adrenodoxin and adrenodoxin reductase that had been isolated from the bovine adrenal NADPH–cytochrome P-450 reductase fraction (1, 12). The purified adrenodoxin and adrenodoxin reductase preparations were completely devoid of cytochrome P-450 as well as hydroxylase activity. The adrenodoxin fraction was devoid of adrenodoxin reductase activity (assayed by reduction of dichlorophenol indophenol (14)) or cytochrome *c* reductase activity and had a ratio between absorbance at 415 nm and 280 nm of 0.4–0.5. Adrenodoxin reductase fraction was devoid of adrenodoxin and NADPH–cytochrome *c* reductase activity and contained 8.5 nmol reductase per mg protein as calculated from the absorbance at 450 nm using an extinction coefficient of 11.3 cm^{-1} mM^{-1} (15).

Preparation of mitochondria, insoluble cytochrome P-450, and NADPH–cytochrome P-450 reductase from rat adrenals

Male rats of the Sprague–Dawley strain weighing about 200 g were used. In the experiments concerning effect of ACTH and potassium chloride, adrenals from groups of 10 rats were used in each experiment as the source of cytochrome P-450. The mitochondrial fraction was prepared by centrifugation from a 1% homogenate (w/v) of adrenals in 0.25 M sucrose containing 0.1 mM dithiothreitol and was washed once by resuspension and recentrifugation in the same buffer (1). The mitochondrial precipitate was suspended in a small amount of distilled water (preparation of submitochondrial fractions) or in 0.1 M Tris-Cl buffer, pH 7.0 (experiments with mitochondria). In incubations with the original mitochondrial fraction, dilution to a protein content of about 0.4 mg/ml was made. After storage of the mitochondrial fraction for 12–24 hr at 4°C, the insoluble cytochrome P-450 and soluble NADPH–cytochrome P-450 reductase fractions were prepared as described previously (1). The recovery of cytochrome P-450 in the insoluble fraction was almost complete as determined with the spectrophotometric method. In 10 different preparations the yield was 98 ± 2% (mean ± SD).

Incubation conditions and assay of incubation products

In the general incubation procedure 80 μg of [1,2-$^3H_2$]deoxycorticosterone (1.5×10^6 cpm), 10 μg of [4-^{14}C]cholesterol (1.5×10^6 cpm), or 10 μg of [4-^{14}C]progesterone (1.5×10^6 cpm) were added to 1.0 ml, 0.5 ml, and 1.0 ml, respectively, of the mitochondrial fraction fortified with 30 μmol of $CaCl_2$ and 3 μmol of NADPH in a total volume of 3 ml of Tris-Cl buffer, pH 7.0 (cf. ref. 1). All the steroids were added dissolved in acetone, 50 μl. In incubations with [1,2-$^3H_2$]corticosterone, 2 μg (1.5×10^6 cpm) were used and the Tris-Cl buffer was substituted with Krebs-Ringer buffer containing 2% glucose (w/v). In incubations with reconstituted systems, 0.1 nmol of the cytochrome P-450 preparations was incubated with 1 or 20 units of NADPH–cytochrome P-450 reductase. In some experiments (Figs. 2 and 3) the NADPH–cytochrome P-450 reductase was replaced with a mixture of purified adrenodoxin (0.4 mg) and adrenodoxin reductase (1.0 mg) corresponding to 8 units of cytochrome *c* reductase activity. Optimal conversion was obtained with 20 units of NADPH–cytochrome P-450 reductase. Prior to addition of isotope the incubation mixture was preincubated at 37°C for 5 min.

In general, incubations were performed at 37°C for 10 min (cholesterol) or 20 min (other substrates) with air as the gas phase. In the case of incubations with corticosterone, an atmosphere containing 93.5% oxygen and 6.5% carbon dioxide was used, as this was shown to give optimal conversion.

Under all the above conditions the rate of conversion was linear with time and with mitochondrial fraction or cytochrome P-450, and the enzyme was saturated with substrate. In the experiments with progesterone as an inhibitor of 11β- and 18-hydroxylation of deoxycorticosterone and of deoxycorticosterone as an inhibitor of 11β-hydroxylation of progesterone, suboptimal concentrations of substrate were used (cf. Figs. 2 and 3).

Incubations with cholesterol and progesterone were terminated with chloroform–methanol 2:1 (v/v). Incubations with corticosterone and deoxycorticosterone were terminated with methylene chloride. The incubation products were extracted and assayed by thin-layer chromatography and radioscanning as described previously (1, 13). The yield in the extraction was 90–95% with respect to both the steroid incubated and the hydroxylated products. It was shown by crystallization to constant specific radioactivity with authentic 11β- and 18-hydroxylated deoxycorticosterone that the radioactivity in the appropriate chromatographic zone corresponded to the respective compound. The purity and identity of the chromatographic fractions were also confirmed by repeated thin-layer chromatography and by combined gas-liquid chromatography–mass spectrometry of methoxime–trimethylsilyl derivatives. In the latter procedure an LKB 9000 instrument equipped with an 1.5% SE-30 column was used. The coefficient of variation within the range obtained in most of the different experiments (0.2–10 nmol converted

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× nmol cytochrome P-450^{-1} × min^{-1}) was less than 12% with respect to assay of 11β- and 18-hydroxylase activity (calculated from five parallel incubations in each set of experiments with homogenates giving different degrees of conversion). Conversion lower than 0.2 nmol converted × nmol cytochrome P-450^{-1} × min^{-1} were obtained only in some fractions in Table 6 and Fig. 1. The ratio between 11β- and 18-hydroxylation in 15 different mitochondrial preparations from rat and bovine homogenates was 3.0 ± 0.25 and 6.9 ± 1.25 ($\bar{x}$ ± SD) respectively. Using Student's t test, this difference was significant ($P < 0.005$).

In incubations of the mitochondrial fraction with corticosterone, small amounts of the primary product, 18-hydroxycorticosterone, were converted into aldosterone. The extent of 18-hydroxylation was calculated as the sum of aldosterone and 18-hydroxylated corticosterone. In order to assay conversion of endogenous cholesterol, part of the chloroform extract of incubations with [4-^{14}C]-cholesterol was converted into trimethylsilyl ether and subjected to mass fragmentography as described previously (13). The same technique was used for determination of endogenous cholesterol. The coefficient of variation in these analyses was less than 5% under all the experimental conditions used.

RESULTS

Effect of potassium chloride treatment on catalytic activity of rat adrenal mitochondria and submitochondrial fractions

Table 2 summarizes results of experiments with adrenal mitochondria either from groups of rats given 0.3 M KCl in 5% glucose or from groups of rats given only 5% glucose in drinking water. The treatment with KCl was found to stimulate the 11β-hydroxylation of deoxycorticosterone and progesterone as well as the 18-hydroxylation of deoxycorticosterone and corticosterone. Side chain cleavage of cholesterol was not affected. The yield of insoluble cytochrome P-450 was about twice higher from adrenals of potassium chloride-treated rats than from control rats (Table 1). **Table 3** summarizes results of experiments with insoluble cytochrome P-450 isolated from the two different mitochondrial fractions together with excess bovine NADPH–cytochrome P-450 reductase. The 11β-hydroxylation of deoxycorticosterone and progesterone as well as the 18-hydroxylation of deoxycorticosterone and corticosterone per nmol of cytochrome P-450 was twice higher in incubations with reconstituted systems containing the cytochrome P-450 from potassium chloride-treated rats than in incubations containing cytochrome P-450 from control rats. Side chain cleavage of cholesterol per nmol of cytochrome P-450 was about the same in both groups regardless of the source of the cytochrome P-450.

In order to exclude a possible influence of the origin of NADPH–cytochrome P-450 reductase on the reaction, 1 unit of NADPH-cytochrome P-450 reductase fraction from either control rats or rats treated with KCl was combined with each of the two different types of cytochrome P-450 fractions. The degree of conversion was the same with both preparations of NADPH–cytochrome P-450 reductase and the differences observed were due only to the origin of the cytochrome P-450 fraction. The yield of NADPH–cytochrome P-450 reductase was the same from both mitochondrial fractions (about 10 units).

Effect of ACTH treatment on catalytic activity of rat adrenal mitochondria and submitochondrial fractions

Table 4 summarizes results of experiments with adrenal mitochondria either from groups of rats

TABLE 2. Effect of treatment with potassium chloride on 11β- and 18-hydroxylation and side chain cleavage by rat adrenal mitochondrial fraction

Subcellular Fractions	Hydroxylation of Deoxycorticosterone		Side Chain Cleavage of Cholesterol		Hydroxylation of Corticosterone	Hydroxylation of Progesterone
	18-	11β-	^{12}C	^{14}C	18-	11β-
	nmol converted × (mg of protein)$^{-1}$ × min^{-1}					
Mitochondrial fraction after potassium chloride treatment	3.0 (2.6–3.4)	7.3 (6.2–8.0)	3.0 (2.6–3.3)	3.9 (3.6–4.1)	0.4 (0.3–0.4)	2.6 (2.2–3.1)
Mitochondrial fraction from corresponding control rat	1.2 (1.1–1.3)	3.3 (3.1–3.5)	3.1 (3.0–3.2)	3.9 (3.7–4.1)	0.1 (0.1–0.2)	1.2 (1.0–1.4)

Standard assay conditions were used. The figures are the mean and range of three independent experiments. Analytical variation is given in Experimental section.

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TABLE 3. Effect of potassium chloride on hydroxylation of cholesterol, corticosterone, deoxycorticosterone and progesterone by reconstituted systems

Subcellular Fractions	Hydroxylation of Deoxycorticosterone		Side Chain Cleavage of Cholesterol		Hydroxylation of Corticosterone	Hydroxylation of Progesterone
	18-	11β-	^{12}C	^{14}C	18-	11β-
	nmol converted × (nmol of cytochrome P-450)$^{-1}$ × min^{-1}					
Cytochrome P-450 from KCl-treated rats						
+ bovine NADPH–cytochrome P-450 reductase	6.0 (5.8–6.3)	12.3 (11.5–13.0)	4.4 (4.3–4.4)	1.7 (1.6–1.7)	0.4 (0.3–0.4)	2.9 (2.1–3.8)
+ NADPH–cytochrome P-450 reductase from KCl-treated rats	1.4 (1.2–1.6)	3.2 (2.7–3.6)	2.1 (1.9–2.3)	0.4 (0.4–0.5)		0.5 (0.3–0.7)
+ NADPH–cytochrome P-450 reductase from control rats	1.3 (1.2–1.5)	3.1 (2.4–3.7)	2.0 (1.9–2.1)	0.4 (0.3–0.4)		0.6 (0.4–0.8)
Cytochrome P-450 from control rats						
+ bovine NADPH-cytochrome P-450 reductase	3.2 (3.1–3.4)	6.7 (6.2–7.1)	4.3 (3.9–4.7)	1.7 (1.4–1.9)	0.2 (0.1–0.2)	1.3 (1.1–1.5)
+ NADPH–cytochrome P-450 reductase from KCl-treated rats	0.5 (0.4–0.6)	1.1 (1.1–1.2)	1.9 (1.7–2.2)	0.6 (0.5–0.7)		0.2 (0.2–0.3)
+ NADPH–cytochrome P-450 reductase from control rats	0.6 (0.6–0.6)	1.3 (1.2–1.3)	1.9 (1.9–2.0)	0.5 (0.4–0.6)		0.2 (0.2–0.3)

Standard assay conditions were used. In incubations with bovine and rat NADPH–cytochrome P-450 reductase, 20 and 1 units of reductase, respectively, were used. The figures given are the mean and range of three independent experiments.

treated for 5 days with daily subcutaneous injections of 25 IU of ACTH in 0.9% NaCl or only 0.9% NaCl. Treatment with ACTH was found to stimulate side chain cleavage of exogenous [4-^{14}C]cholesterol. 11β-Hydroxylation of deoxycorticosterone and progesterone and 18-hydroxylation of deoxycorticosterone and corticosterone were not affected by treatment with ACTH. **Table 5** summarizes results of experiments with insoluble cytochrome P-450 isolated from the two different preparations of mitochondria.

Side chain cleavage of exogenous cholesterol per nmole of cytochrome P-450 was higher in incubations with reconstituted systems containing cytochrome P-450 from ACTH-treated rats than in those containing cytochrome P-450 from control rats. The rate of side chain cleavage of endogenous cholesterol was, however, not affected by the ACTH treatment. 11β-Hydroxylation of deoxycorticosterone and progesterone as well as 18-hydroxylation of deoxycorticosterone and corticosterone per nmol of cytochrome P-450 also were not affected by the treatment with ACTH. The yield of insoluble cytochrome P-450 was about the same from both mitochondrial fractions, about 6 nmol (Table 1). The content of cholesterol was however decreased about 60% in the preparation obtained from the ACTH-treated rats.

TABLE 4. Effect of treatment of ACTH on 11β- and 18-hydroxylation and side chain cleavage by rat adrenal mitochondrial fraction

	Hydroxylation of Deoxycorticosterone		Side Chain Cleavage of Cholesterol		Hydroxylation of Corticosterone	Hydroxylation of Progesterone
	18-	11β-	^{12}C	^{14}C	18-	11β-
	nmol converted × (mg of protein)$^{-1}$ × min^{-1}					
Mitochondrial fraction after ACTH-treatment	1.3 (1.1–1.5)	3.0 (2.7–3.5)	4.7 (4.4–5.0)	7.8 (7.2–8.1)	0.2 (0.1–0.2)	1.3 (1.0–1.5)
Mitochondrial fraction from corresponding control rats	1.7 (1.6–1.9)	3.3 (3.0–3.4)	2.9 (2.4–3.2)	3.9 (3.7–4.1)	0.2 (0.1–0.2)	1.1 (1.0–1.2)

Standard assay conditions were used. The figures given are the mean and range of three independent experiments.

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TABLE 5. Effect of ACTH on hydroxylation of cholesterol, corticosterone, deoxycorticosterone, and progesterone by reconstituted systems

Subcellular Fractions	Hydroxylation of Deoxycorticosterone		Side Chain Cleavage of Cholesterol		Hydroxylation of Corticosterone	Hydroxylation of Progesterone
	18-	11β-	^{12}C	^{14}C	18-	11β-
	nmol converted × (nmol of cytochrome P-450)$^{-1}$ × min^{-1}					
Cytochrome P-450 from ACTH-treated rats						
+ bovine NADPH–cytochrome P-450 reductase (20 units)	4.2 (3.8–4.7)	8.8 (8.3–9.6)	5.1 (4.9–5.2)	3.7 (3.1–4.2)	0.1 (0.1–0.1)	1.3 (1.1–1.4)
+ NADPH–cytochrome P-450 reductase from ACTH-treated rats (1 unit)	0.6 (0.5–0.8)	1.4 (1.3–1.5)	1.9 (1.6–2.1)	0.9 (0.8–1.0)		0.3 (0.2–0.3)
+ NADPH–cytochrome P-450 reductase from control rats (1 unit)	0.6 (0.6–0.7)	1.2 (1.1–1.4)	1.9 (1.8–2.0)	0.8 (0.7–0.9)		0.3 (0.3–0.4)
Cytochrome P-450 from control rats						
+ bovine NADPH–cytochrome P-450 reductase (20 units)	3.9 (3.6–4.4)	7.9 (7.4–8.9)	4.8 (4.6–5.1)	1.9 (1.8–1.9)	0.1 (0.1–0.2)	1.3 (1.2–1.3)
+ NADPH–cytochrome P-450 reductase from ACTH-treated rats (1 unit)	0.6 (0.5–0.7)	1.2 (1.1–1.2)	1.2 (1.1–1.3)	1.7 (1.4–2.1)	0.5 (0.4–0.5)	0.2 (0.2–0.3)
+ NADPH–cytochrome P-450 reductase from control rats (1 unit)	0.6 (0.5–0.7)	1.2 (1.1–1.3)	1.7 (1.4–2.1)	0.5 (0.4–0.5)		0.2 (0.2–0.3)

Standard assay conditions were used. In incubations with bovine and rat NADPH–cytochrome P-450 reductase, 20 and 1 units of reductase, respectively, were used. The figures given are the mean and range of three independent experiments.

Experiments similar to those described above, for the effect of potassium chloride treatment, excluded the possibility that the reaction was influenced by the origin of the NADPH–cytochrome P-450 reductase and showed that observed differences were due only to the origin of the cytochrome P-450 fraction.

Hydroxylase activities in subfractionated soluble cytochrome P-450 preparations

A reconstituted system consisting of soluble cytochrome P-450 from either rat or bovine adrenals and excess bovine NADPH–cytochrome P-450 reductase was active towards deoxycorticosterone, progesterone, and cholesterol. There was no significant activity towards corticosterone (cf. ref. 1). The most obvious difference between the two preparations of cytochrome P-450 was the high ratio between 11β- and 18-hydroxylase activity in the system containing bovine cytochrome P-450 (cf. ref. 1). The ratio between 11β-hydroxylation of deoxycorticosterone and progesterone was sometimes found to be higher in the soluble system as compared with insoluble systems.

Soluble cytochrome P-450 fractions from the two species were subjected to gel chromatography on a Sephadex G-200 column. In both cases, protein as well as cytochrome P-450 were eluted as a broad peak (**Fig. 1**). An aliquot of each chromatographic

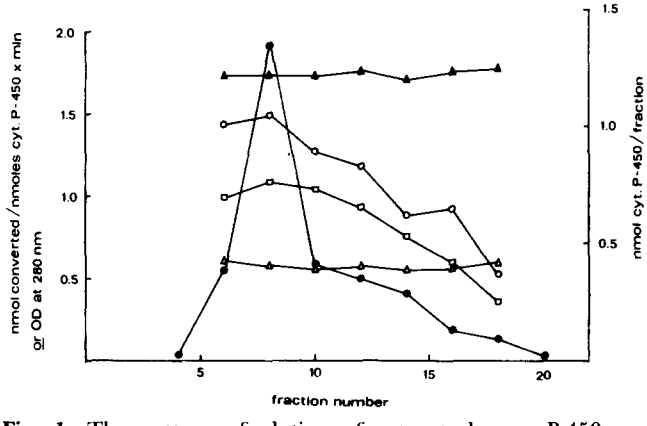

Fig. 1 The pattern of elution of rat cytochrome P-450 on Sephadex G-200. Cytochrome P-450 (0.1 nmol) was added to 0.4 mg adrenodoxin and 1.0 mg of adrenodoxin reductase and incubated with [4-^{14}C]cholesterol (10 μg) or [1,2-$^{3}H_2$]deoxycorticosterone (40 μg) under standard incubation conditions. ● —— ●, O.D. at 280 nm; □ —— □, side chain cleavage activity; ▲ —— ▲, 11β-hydroxylation, and △ —— △, 18-hydroxylation of deoxycorticosterone: ○ —— ○, nmoles cytochrome P-450 per fraction. For further details, see Experimental Procedure and Table 6.

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fraction corresponding to 0.1–0.3 nmol of cytochrome P-450 was incubated with adrenodoxin, adrenodoxin reductase, and the different substrates. The 18- and 11β-hydroxylase activity per nmol of cytochrome P-450 was about constant in the different chromatographic fractions (Table 6 and Fig. 1). The cholesterol side chain cleavage activity per nmol of cytochrome P-450 was, however, considerably higher in the early chromatographic fractions than in the late ones.

The crude NADPH–cytochrome P-450 reductase fraction from bovine adrenals that contained small amounts of cytochrome P-450 (see Materials and Methods) was subjected to gel chromatography as above (bovine cytochrome P-450, preparation 2 in Table 6). 18- and 11β-Hydroxylase activities per nmol of cytochrome P-450 remained constant in all chromatographic fractions. Cholesterol side chain cleavage activity per nmol of cytochrome P-450 was, however, about five times higher in the early than in the late chromatographic fractions.

Inhibition of 11β- and 18-hydroxylation of [1,2-$^3H_2$]deoxycorticosterone by progesterone

Fig. 2 summarizes results of experiments in which progesterone was used as an inhibitor of 11β- and 18-hydroxylation of [1,2-$^3H_2$]deoxycorticosterone. The inhibition appeared to be of a noncompetitive nature in the case of both 11β- and 18-hydroxylation of deoxycorticosterone when using crude rat adrenal mitochondria as the source of enzyme (Fig. 2A and 2B). When using insoluble or soluble cytochrome P-450 from rat adrenals, however, the inhibition was apparently of a competitive nature in the case of both 11β- and 18-hydroxylation of deoxycorticosterone (Fig. 2C–2F). The K_m for the 11β- as well as for the 18-hydroxylase activity in the soluble system was about 2×10^{-5} M.

Inhibition of 11β-hydroxylation of [4-^{14}C]progesterone by deoxycorticosterone

Fig. 3 summarizes results of experiments in which deoxycorticosterone was used as an inhibitor of 11β-hydroxylation of [4-^{14}C]progesterone. The inhibition appeared to be of a noncompetitive nature when using mitochondria as source of enzyme (Fig. 3A) but of a competitive nature when using insoluble or soluble cytochrome P-450 (Fig. 3B, 3C). The K_m for 11β-hydroxylation of progesterone in the soluble system was about 1.3 ± 10^{-5} M.

DISCUSSION

Effect of treatment with potassium chloride and ACTH

In agreement with the previous work by Baumann and Müller (3), adrenal mitochondrial 18-hydroxyla-

TABLE 6. Subfractionation of soluble cytochrome P-450 preparations by Sephadex chromatography

Fractions from Column	nmol Cytochrome P-450/mg Protein	Hydroxylation of Deoxycorticosterone 18-	Hydroxylation of Deoxycorticosterone 11β-	Hydroxylation of Cholesterol ^{14}C	Ratio 11β-/SCC[a]	Ratio 11β-/18-
	nmol converted × (nmol cytochrome P-450)$^{-1}$ × min^{-1}					
Soluble rat cytochrome P-450						
Before chromatography	2.4	0.4	1.6	1.2	1.3	4.0
Fraction 6	1.6	0.6	1.7	1.1	1.5	2.8
Fraction 16	4.6	0.6	1.8	0.6	3.0	3.0
Soluble bovine cytochrome P-450 (Preparation I)						
Before chromatography	2.2	0.2	1.0	1.3	0.8	5.0
Fraction 6	1.8	0.2	0.8	1.0	0.8	4.0
Fraction 16	1.1	0.2	0.8	0.4	2.0	4.0
Soluble bovine cytochrome P-450 (Preparation II)						
Before chromatography	1.1	0.01	0.07	0.4	0.2	7.0
Fraction 6	1.3	0.02	0.10	0.5	0.2	5.0
Fraction 16	3.3	0.02	0.10	0.1	1.0	5.0

[a] SCC, side chain cleavage.

Standard assay conditions with 0.4 mg of adrenodoxin and 1.0 mg of adrenodoxin reductase were used. In incubations with cholesterol and deoxycorticosterone, 10 and 40 μg, respectively, of substrate were used. The incubation mixtures contained less than 0.05 μg of unlabeled cholesterol prior to incubation. The total recovery of cytochrome P-450 in the chromatography of the rat and bovine preparation was about 80% and 85%, respectively.

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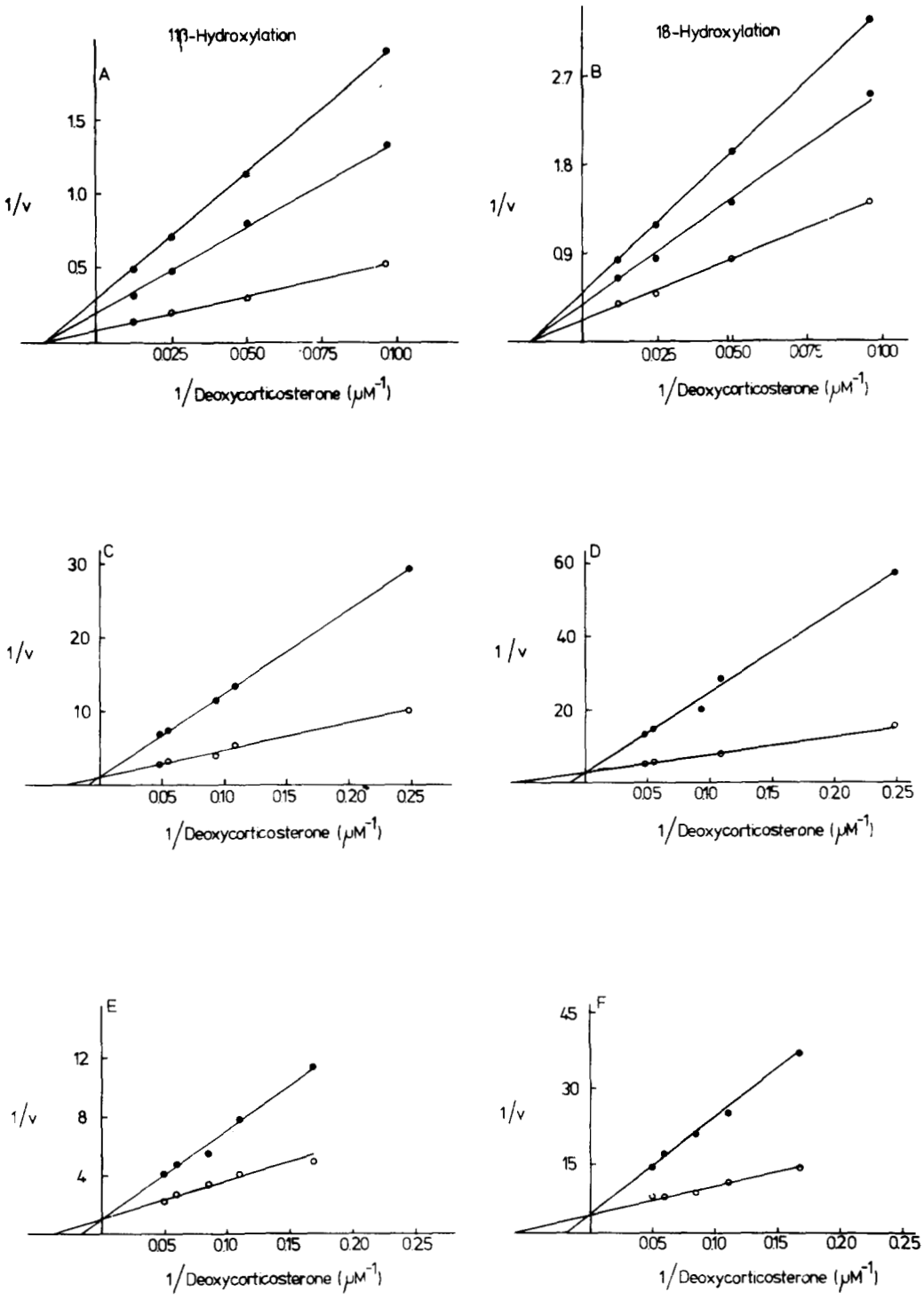

Fig. 2. Effect of progesterone on 11β- and 18-hydroxylation of [1,2-$^3H_2$]deoxycorticosterone in mitochondria (A, B), insoluble (C, D) and soluble (E, F) cytochrome P-450 systems. Mitochondrial fraction (corresponding to 0.2 nmol cytochrome P-450), insoluble and soluble cytochrome P-450 (0.1 nmol), adrenodoxin (0.4 mg), and adrenodoxin reductase (1.0 mg) were incubated with labeled and unlabeled steroid in equimolar amounts. Standard assay conditions were used. Rate of hydroxylation (v) is expressed as nmol converted substrate × nmol cytochrome P-450^{-1} × min^{-1}. Lines were drawn using the method of least squares.

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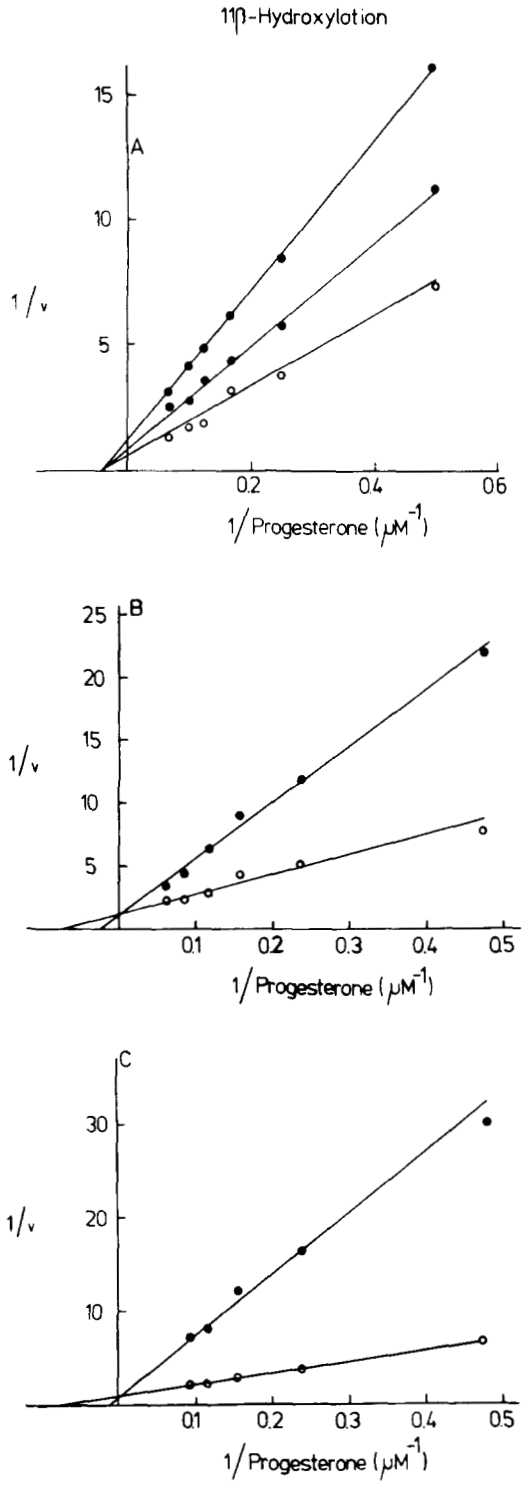

Fig. 3. Effect of deoxycorticosterone on 11β-hydroxylation of [4-^{14}C]progesterone in mitochondria (A), insoluble (B) and soluble (C) cytochrome P-450 systems. Mitochondrial fraction (corresponding to 0.2 nmol cytochrome P-450), insoluble and soluble cytochrome P-450 (0.1 nmol), adrenodoxin (0.4 mg) and adrenodoxin reductase (1.0 mg) were incubated with labeled and unlabeled steroid in equimolar amounts. Standard assay conditions were used. Rate of hydroxylation (v) is expressed as nmol converted substrate × nmol cytochrome P-450^{-1} × min^{-1}. Lines were drawn using the method of least squares.

tion of corticosterone increased by about 100% after treatment of rats with KCl. 18-Hydroxylation of deoxycorticosterone and 11β-hydroxylation of deoxycorticosterone and progesterone increased to about the same extent, whereas cholesterol side chain cleavage was unaffected. It was shown that the increased 18- and 11β-hydroxylase activities were due to increased amounts of cytochrome P-450 specific for these hydroxylations. The cytochrome P-450 isolated from mitochondrial fractions of potassium chloride-treated rats had higher 18- and 11β-hydroxylase activities per nmol of cytochrome P-450 than that isolated from mitochondrial fractions of control rats. The increase in 18- and 11β-hydroxylase activity per nmol of cytochrome P-450 in the crude mitochondrial fraction from KCl-treated rats was however less than could be calculated from the increase in concentration of cytochrome P-450 and the increase in activity per nmol of cytochrome P-450 in the reconstituted system.

It is concluded that the amount of specific cytochrome P-450 in the isolated mitochondrial fraction is not the only limiting factor for 18- and 11β-hydroxylase activity. It is probable that the mitochondrial fraction has a relative deficiency of NADPH– cytochrome P-450 reductase activity. As pointed out in the previous work (1), some adrenodoxin is lost during preparation of the mitochondrial fraction. The cholesterol side chain cleavage activity per nmol of cytochrome P-450 was about the same in both preparations of cytochrome P-450. There was no increase in amount of NADPH-cytochrome P-450 reductase and there were no differences in specific catalytic activity of the NADPH–cytochrome P-450 reductase prepared from mitochondrial fraction of potassium chloride-treated rats and from control rats. Long-term treatment with ACTH increased mitochondrial cholesterol side chain cleavage activity, whereas 18- and 11β-hydroxylation were unaffected. The increase in side chain cleavage of exogenous cholesterol was more marked than that of endogenous cholesterol. This might be due to a depletion of endogenous substrate since the mitochondrial fraction of ACTH-treated rats had a cholesterol content only half that of the mitochondrial fraction of untreated rats. The increase in cholesterol side chain cleavage activity was probably due to increased amounts of the specific type of cytochrome P-450 involved in this activity, since the cytochrome P-450 isolated from ACTH-treated rats had a higher activity per nmol of cytochrome P-450 than that isolated from untreated rats. The 18- and 11β-hydroxylase activity per nmol of cytochrome P-450 was about the same in both preparations of

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cytochrome P-450. The yield and the specific catalytic activity of NADPH–cytochrome P-450 reductase were the same from mitochondrial fractions of ACTH-treated animals and untreated animals. It must be pointed out that, in the above considerations, "exogenous" and "endogenous" cholesterol refer only to labeled and unlabeled cholesterol, respectively. It cannot be precluded that endogenous cholesterol behaves like a single pool different from the exogenous substrate. There is evidence that there is a progressive equilibrium between exogenous and endogenous cholesterol up to a certain point; part of the endogenous cholesterol does not seem to be fully equilibrated (13). The possibility that side chain cleavage of exogenous and endogenous cholesterol is catalyzed by different enzymes has however not been completely excluded. If so, ACTH might affect the two systems differently.

Experiments with subfractionated soluble cytochrome P-450

Shikita, Hall, and Isaka (6) and Shikita and Hall (17, 18) have described purification of a species of soluble bovine adrenal cytochrome P-450 involved in cholesterol side chain cleavage. This cytochrome P-450, purified almost to homogeneity, has a molecular weight of about 850,000 and consists of up to 16 subunits. The preparation contained some 11β-hydroxylase activity and the ratio between cholesterol side chain cleavage activity and 11β-hydroxylase activity was about 10. Schleyer, Cooper, and Rosenthal (8) have described partial purification of bovine adrenal cytochrome P-450 involved in 11β-hydroxylation by a somewhat different procedure. The preparation also contained cholesterol side chain cleavage activity. Shikita et al. (6, 17) have reported that their preparation contained no 18-hydroxylase activity. The analytical limit was, however, not defined and it must be borne in mind that the ratio between 11β- and 18-hydroxylase activity in bovine adrenal mitochondria with deoxycorticosterone as substrate varies between 5 and 10 (cf. 1). There is no information concerning the presence of 18-hydroxylase activity in the cytochrome P-450 prepared by Schleyer et al. (8). In the present work, a soluble cytochrome P-450 fraction was prepared from bovine adrenals essentially according to Schleyer et al. (8). The preparation had about the same purity with respect to cytochrome P-450 per mg protein as that described by Schleyer et al. (8). The preparation contained 18- and 11β-hydroxylase activity as well as cholesterol side chain cleavage activity. The ratio between 11β- and 18-hydroxylase activity on deoxycorticosterone was similar in the soluble fraction and in the insoluble and crude mitochondrial fractions. The ratio between 11β-hydroxylation of deoxycorticosterone and progesterone was sometimes 2–4 times lower with the soluble fraction as compared to the insoluble fraction. This might be due to the presence of different 11β-hydroxylating systems that are separated during the purification. Another possibility is that the purification procedure changes the properties of the enzyme system. Gel filtration of the soluble preparation was found to change the ratio between 11β-hydroxylase activity and cholesterol side chain cleavage by a factor of about 2 without significant change in the ratio between 18- and 11β-hydroxylase activity. The partial separation between 11β-hydroxylase activity and cholesterol side chain cleavage activity on the Sephadex G-200 column might be due to different states of aggregation of the two species of cytochrome P-450.

The procedures used in the present investigation, which in essence represent a combination of those described by Shikita and Hall (17, 18) and by Schleyer et al. (8), gave cytochrome P-450 preparations from bovine adrenals with a high ratio between 11β-hydroxylase activity and cholesterol side chain cleavage activity. The crude bovine NADPH–cytochrome P-450 reductase fraction prepared according to Schleyer et al. (8), was found to contain small amounts of cytochrome P-450 with a high ratio between cholesterol side chain cleavage activity and 18- and 11β-hydroxylase activity. Gel chromatography of this fraction changed the ratio between 11β-hydroxylase activity and cholesterol side chain cleavage activity in the same way as above without affecting the ratio between 18- and 11β-hydroxylase activity.

As pointed out previously (1), bovine cytochrome P-450 is less suited to studies of 18-hydroxylation in view of the high ratio between 11β- and 18-hydroxylase activity. Soluble cytochrome P-450 was therefore prepared also from rat adrenal mitochondria. The ratio between the different activities in this soluble fraction was about the same as in the mitochondrial fraction. Sephadex chromatography resulted in changes between the different catalytic activities similar to those found in the corresponding experiments with bovine adrenal cytochrome P-450.

Inhibition experiments

The constant ratio between 11β- and 18-hydroxylation of deoxycorticosterone during the different in vivo conditions, and after partial inactivation or purification, is not conclusive per se for the hypothesis that the cytochrome P-450 involved in 18-hydroxyla-

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tion of deoxycorticosterone is identical with that involved in 11β-hydroxylation of the same substrate. The inhibition experiments performed with soluble rat adrenal cytochrome P-450, however, strongly support the contention that the same site on cytochrome P-450 is involved in 18- and 11β-hydroxylation of deoxycorticosterone in rat adrenals. Thus, progesterone competitively inhibited 18- and 11β-hydroxylation of deoxycorticosterone to the same degree and deoxycorticosterone competitively inhibited 11β-hydroxylation of progesterone. Interestingly, the inhibition was found to be of a noncompetitive nature when using a crude mitochondrial fraction as the source of enzyme. Most probably this reflects different transport mechanisms for entrance of the different steroids into the mitochondrion. It should be pointed out that the apparent K_m values for the different activities were higher in the experiments with the crude mitochondrial fraction than in the experiments with soluble cytochrome P-450. The inhibition experiments do not exclude the presence of different types of cytochrome P-450 active towards deoxycorticosterone, each type with a common characteristic site for 18- and 11β-hydroxylation. If such different types of cytochrome P-450 species exist, they may all give different ratios between 11β- and 18-hydroxylase activity. With the possible exception of the experiments by Shikita et al. (6, 17), there is as yet no conclusive evidence for the existence of several types of cytochrome P-450 active towards deoxycorticosterone. At the present state of knowledge, it also can not be excluded that specific compounds may interfere with cytochrome P-450 and affect the ratio between 18- and 11β-hydroxylation of deoxycorticosterone.

Recently, Rapp and Dahl (19) also concluded that the same species of cytochrome P-450 is involved in both 18- and 11β-hydroxylation of deoxycorticosterone in rats. These authors showed that rats, bred for their blood pressure response to a high salt diet, showed a Warburg partition constant (CO/O_2 ratio) that was constant for 18- and 11β-hydroxylation of deoxycorticosterone for each strain tested. Considerable differences were observed, however, between different strains of rats tested. The between-strain difference was unique for 18- and 11β-hydroxylation and was not seen for cholesterol side chain cleavage or 21-hydroxylation. Our results strongly support the conclusion drawn by Rapp and Dahl (19). It is important to point out that the results of the present work, as well as the work by Rapp and Dahl, only give evidence for a homogeneity of the enzyme system in rat adrenal preparations and with deoxycorticosterone and progesterone as substrates. The results of the experiments with bovine adrenal preparations obtained in the present work were similar to those obtained with rat adrenal preparations. The relatively low 18-hydroxylase activity of the bovine preparations, however, prevented inhibition studies of sufficient accuracy.

In view of the specific role of the 18-hydroxylase activity for mineralcorticoid function, it would be surprising if the same species of cytochrome P-450 is involved in this hydroxylation as in 11β-hydroxylation. It should be pointed out, however, that the biological importance of 18-hydroxydeoxycorticosterone is uncertain. The mineralcorticoid effect of this steroid is less than that of aldosterone. 18-Hydroxydeoxycorticosterone seems to be excreted as such from the adrenals and is probably of little importance as an intermediate in the biosynthesis of aldosterone, at least in rats. The major substrate for 18-hydroxylase activity in the biosynthesis of aldosterone is most probably corticosterone. Our results do not exclude that the 18-hydroxylase involved in biosynthesis of aldosterone is different from that studied in the present work. In fact, results of recent work with different inhibitors of 11β-hydroxylation of deoxycorticosterone and 18-hydroxylation of corticosterone seem to suggest that different enzymes are involved in these hydroxylations (20).

In view of the low 18-hydroxylase activity towards corticosterone in reconstituted systems (cf. Tables 3 and 4) it was not possible to characterize this system in detail in the present work. The adrenal preparations used contained all the different adrenal zones. Conversion of corticosterone into 18-hydroxycorticosterone is, at least by some authors, believed to be most efficient in zona glomerulosa (21). Furthermore, it has been suggested that 18-hydroxylation of endogenous corticosterone occurs by a mechanism different from that of exogenous corticosterone (22). Attempts in our laboratory to study specifically 18-hydroxylation of endogenous corticosterone in adrenal preparations by use of mass fragmentography has failed to date.

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