

Assay and properties of 18-hydroxylation of endogenous and exogenous corticosterone in rat adrenals. Evidence for heterogeneity of 18-hydroxylase activity.

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Abstract A mass fragmentographic technique for assay of 18-hydroxylation of labeled (exogenous) and unlabeled (endogenous) corticosterone in adrenal mitochondria and in reconstituted cytochrome P-450 systems has been developed. An extract of an incubation of [4-¹⁴C]corticosterone is subjected both to thin-layer radiochromatography and to mass fragmentography (as *O*-methyloxime-trimethylsilyl ether derivative). In the latter procedure the ions at *m/e* 605 and 607 (specific for the derivatives of unlabeled and labeled 18-hydroxycorticosterone, respectively), at *m/e* 591 and 593 (specific for the derivatives of unlabeled and labeled aldosterone, respectively) and at *m/e* 548 and 550 (specific for the derivatives of unlabeled and labeled corticosterone, respectively) were followed through the gas-liquid chromatography. From the ratio between the peaks obtained in the mass fragmentography and from the percentage conversion of [4-¹⁴C]corticosterone obtained in the thin-layer radiochromatography, the amount of endogenous and exogenous 18-hydroxycorticosterone and aldosterone could be calculated. The effects of time, enzyme, and substrate concentration of 18-hydroxylation were studied and optimal conditions for assay were determined. Under most conditions, the ratio between labeled and unlabeled 18-hydroxylated products was about constant, indicating that labeled and unlabeled corticosterone were not in equilibrium. It was ascertained that the 18-hydroxycorticosterone and aldosterone formed in the incubations were derived from corticosterone. [4-¹⁴C]18-Hydroxydeoxycorticosterone was not converted into aldosterone or 18-hydroxycorticosterone. In vitro studies with different 18-hydroxylase inhibitors (spironolactone, canrenone, and canrenoate-K) and studies with rats pretreated with KCl in drinking fluid suggest that 18-hydroxylation of corticosterone is catalyzed by an enzyme system different from that catalyzing 18-hydroxylation of deoxycorticosterone. — **Karlmar, K-E.** Assay and properties of 18-hydroxylation of endogenous and exogenous corticosterone in rat adrenals. Evidence for heterogeneity of 18-hydroxylase activity. *J. Lipid Res.* 1979. **20:** 729–739.

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Most previous studies on 18-hydroxylation of different steroids in mitochondrial or submitochondrial adrenal preparations have been based on the conver-

sion of added labeled (exogenous) steroids (1–6). Thus the conversion of endogenous steroids has not been taken into account. With some substrates, the conversion of endogenous steroids is negligible as compared to the conversion of the added steroid. Thus, at substrate saturation level, more than 90% of 18-hydroxylation of deoxycorticosterone occurs with the added substrate (5). In studies on 18-hydroxylation of corticosterone, however, the endogenous substrate seems to be more important. It has even been suggested that endogenous and exogenous corticosterone might be hydroxylated by different mechanisms in adrenal preparations (7, 8).

Conventional methods for the study of conversion of unlabeled steroids in adrenals are laborious and complex due to problems with specificity and to the small amount of steroid present. Radioimmunoassay may be an alternative (9), but only a few of the commercially available steroid antibodies have sufficient specificity for use in crude adrenal homogenates prior to elaborate extraction and purification procedures.

In a previous work from this laboratory, a mass fragmentographic technique of high specificity was described (10). This technique allowed determination of rate of hydroxylation of both labeled and unlabeled cholesterol in adrenal homogenates under optimal assay conditions. In the present work a similarly accurate technique has been applied to studies on 18-hydroxylation of exogenous and endogenous cortico-

Abbreviations: MO-TMS, *O*-methyloxime-trimethylsilyl ether. The following nomenclature is used: aldosterone, 11 β ,21-dihydroxy-3,20-diketo-4-pregnen-18-al; corticosterone, 11 β ,21-dihydroxy-4-pregnen-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnen-3,20-dione; 18-hydroxycorticosterone, 11 β ,18,21-trihydroxy-4-pregnen-3,20-dione; 18-hydroxydeoxycorticosterone, 18,21-dihydroxy-4-pregnen-3,20-dione; spironolactone, 3-(3-oxo-7 α -acetylthio-17 β -hydroxy-4-androsten-17 α -yl)propionic acid lactone; canrenone, 3-(3-oxo-17 β -hydroxy-4,6-androstadien-17 α -yl)propionic acid lactone; canrenoate-K, potassium-3-(3-oxo-17 β -hydroxy-4,6-androstadien-17 α -yl)propionate.

sterone in rat adrenal preparations. In view of the possibility that 18-hydroxylation of corticosterone and deoxycorticosterone may be catalyzed by different enzymes (5, 11), both these activities have been assayed under different experimental conditions.

MATERIALS

[4-¹⁴C]Corticosterone (sp act 52 $\mu\text{Ci}/\mu\text{mol}$) and [1,2-³H₂]deoxycorticosterone (sp act 10 $\mu\text{Ci}/\mu\text{mol}$) were purchased from Radiochemical Centre (Amersham, England). The purity of the labeled compounds was ascertained by thin-layer chromatography followed by radioactivity scanning of the chromatoplates (cf. below). [4-¹⁴C]18-Hydroxydeoxycorticosterone (26 $\mu\text{Ci}/\mu\text{mol}$) was prepared by incubation of [4-¹⁴C]deoxycorticosterone of high specific radioactivity (55 $\mu\text{Ci}/\mu\text{mol}$) with rat adrenal mitochondrial fraction. The material was purified by several thin-layer chromatographic processes and contained <0.5% corticosterone, 18-hydroxycorticosterone, or aldosterone when assayed by radio gas-liquid chromatography and gas-liquid chromatography-mass spectrometry.

Aldosterone, corticosterone, deoxycorticosterone, spironolactone, and isocitric dehydrogenase (type IV) were obtained from Sigma Chemical Corp. (St. Louis, MO). The enzyme solution contained 10 mg of protein per ml, and 1 mg of the protein converted 4 nmol of isocitrate into α -ketoglutarate per min. Canrenone and canrenoate-K were generous gifts from G. D. Searle Co. (Chicago, IL.). 18-Hydroxycorticosterone and 18-hydroxydeoxycorticosterone were purchased from Steraloids (Pawling, NY). Sephadex G-100 and DEAE-cellulose were obtained from Pharmacia (Uppsala, Sweden) and Whatman (Maidstone, England), respectively.

METHODS

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

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 $\text{cm}^{-1} \text{mM}^{-1}$ (12). NADPH-cytochrome P-450 reductase activity was assayed according to Masters, Williams, and Kamin (13) and was expressed in units (one unit equals reduction of 1 nmol of cytochrome *c* per min). Protein was determined according to Lowry et al. (14).

Animal conditions and preparation of adrenal mitochondria and submitochondrial fractions

Male rats of the Sprague-Dawley strain weighing about 200 g were used. In the experiments with potassium chloride treatment, groups of rats were given 0.15–0.45 mol/l potassium chloride in 5% (w/v) glucose or only glucose solution as drinking fluid for up to 19 days (cf. 5). As previously shown, this treatment did not change potassium or sodium concentration in serum at the time of killing (8 AM) (15, 16).

Mitochondrial fractions and insoluble cytochrome P-450 from rat adrenals, crude bovine NADPH-cytochrome P-450 reductase (containing a mixture of adrenodoxin and adrenodoxin reductase), as well as partially purified adrenodoxin and adrenodoxin reductase were prepared as described previously (4) from 1% (w/v) adrenal homogenates. The protein content of the mitochondrial fraction was about 0.4 mg/ml. The isolated insoluble cytochrome P-450 fraction contained about 98% of the amount of cytochrome P-450 present in the original mitochondrial fraction (cf. 5, 6).

Incubation conditions

In the standard incubation procedure, 5 μg of [4-¹⁴C]corticosterone dissolved in 25 μl of acetone was added to 1.5 ml of the rat mitochondrial fraction fortified with an NADPH generating system (0.03 μmol of MnCl_2 , 3 μmol of NADP^+ , 12.5 μmol of isocitrate, and 15 μl of isocitric dehydrogenase solution) in a total volume of 3 ml of Krebs-Ringer solution containing 2% (w/v) glucose. In incubations with [1,2-³H₂]deoxycorticosterone, 80 μg of substrate, dissolved in 25 μl of acetone, was added to 1 ml of the mitochondrial fraction fortified with 30 μmol of CaCl_2 and 3 μmol of NADPH in a total volume of 3 ml of 0.1 M Tris-Cl buffer, pH 7.0 (cf. 4). In incubations of [4-¹⁴C]corticosterone with reconstituted cytochrome P-450 systems, the mitochondrial fraction was replaced with 0.3–0.4 nmol of insoluble rat cytochrome P-450 and 40 units of bovine NADPH-cytochrome P-450 reductase. Prior to the addition of isotope, all incubations were preincubated at 37°C for 5 min. Spironolactone, canrenone, and canrenoate-K were added to the incubation mixture prior to preincubation. Incubations with deoxycorticosterone were performed for 20 min with air as the gas phase. Corticosterone incubations were performed in an atmosphere containing 93.5% oxygen and 6.5% carbon dioxide for 30 min. In the inhibition studies, [1,2-³H₂]deoxycorticosterone was incubated under conditions identical with those for incubation of corti-

costerone (cf. below and Fig. 6). Suboptimal amounts of substrate were used in these experiments but the rate of conversion was still linear with concentration of enzyme and time. The conversion of [1,2-³H₂]deoxycorticosterone was about 30% lower in the Krebs-Ringer buffer than in the Tris-Cl buffer. The ratio between 18- and 11 β -hydroxylation of deoxycorticosterone was the same with both buffers. Incubations of [4-¹⁴C]corticosterone under conditions identical with those used for standard incubations of [1,2-³H₂]deoxycorticosterone (with Tris-Cl buffer) in general gave lower degrees of conversion and less reproducible results.

Analysis of incubation products

To assay the extent of conversion of [4-¹⁴C]corticosterone, part of a methylene chloride extract was subjected to thin-layer chromatography and radioscanning as described previously (4, 5). Under the chromatographic conditions employed there was sufficient separation between corticosterone, 18-hydroxycorticosterone, and aldosterone. In some incubations, small amounts of aldosterone had been formed. In these cases the extent of 18-hydroxylation was calculated as the sum of aldosterone and 18-hydroxycorticosterone. Part of the extract was converted into *O*-methyloxime-trimethylsilyl ether (MO-TMS) (17) and analyzed by combined gas-liquid chromatography-mass spectrometry using an LKB 9000 instrument equipped with a MID unit (multiple ion detector). Conditions of the assay and instrument settings were the same as described previously for the assay of cholesterol side-chain cleavage (10) except that column temperature was 265–270°C. The channels of the MID unit were focused at *m/e* 548 (molecular ion of the MO-TMS derivative of unlabeled corticosterone), *m/e* 550 (molecular ion of the MO-TMS derivative of labeled corticosterone), *m/e* 605 (*M* – 31 ion of the MO-TMS derivative of unlabeled 18-hydroxycorticosterone) and *m/e* 607 (*M* – 31 ion of the MO-TMS derivative of labeled 18-hydroxycorticosterone). When the content of aldosterone in the methylene chloride extract was assayed, channels 1 and 2 were focused at *m/e* 591 (molecular ion of the MO-TMS derivative of unlabeled aldosterone) and *m/e* 593 (molecular ion of the MO-TMS derivative of labeled aldosterone), respectively. The amplification factor of the pair of channels measuring labeled and unlabeled molecules of each compound were always set the same. The peak height of the recordings was measured as this was found to give more reproducible results than measurements of peak area. In connection with the estimation of endogenous corticosterone and 18-

hydroxydeoxycorticosterone prior to incubation, a small amount of [4-¹⁴C]corticosterone or [4-¹⁴C]18-hydroxydeoxycorticosterone was added to the enzyme fraction prior to extraction. Further analysis was performed as described above.

Incubations with [1,2-³H₂]deoxycorticosterone were extracted and assayed by thin-layer chromatography and radioscanning as described previously (4).

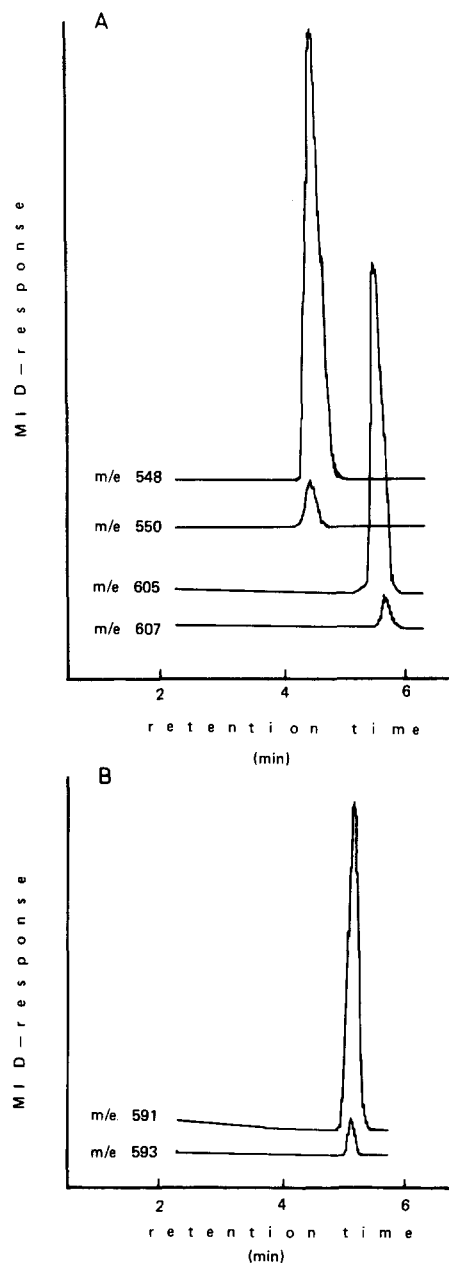


Fig. 1. MID recordings of unlabeled MO-TMS derivatives. *A* shows the MID recording of the MO-TMS derivative of unlabeled corticosterone (*m/e* 548 and 550) and 18-hydroxycorticosterone (*m/e* 605 and 607). *B* shows the MID recording of the MO-TMS derivatives of aldosterone (*m/e* 591 and 593). For further details, see Methods.

Calculations

The amount of 4-¹⁴C-labeled corticosterone after incubation, P_{ex} , was calculated by radioscanning of the thin-layer plate. The amount of endogenous unlabeled corticosterone after incubation, P_{en} , could be calculated from the following equation

$$\frac{I_{548}}{I_{550}} = \frac{P_{ex} \left[\frac{I_{548,^{14}C}}{I_{548,^{14}C} + I_{550,^{14}C}} \right] + P_{en} \left[\frac{I_{548,^{12}C}}{I_{548,^{12}C} + I_{550,^{12}C}} \right]}{P_{ex} \left[\frac{I_{550,^{14}C}}{I_{548,^{14}C} + I_{550,^{14}C}} \right] + P_{en} \left[\frac{I_{550,^{12}C}}{I_{548,^{12}C} + I_{550,^{12}C}} \right]}$$

In this equation, I_{548} and I_{550} correspond to the peak height of the tracings at m/e 548 and 550, respectively, in the MID recording of the derivative of the extract. $I_{548,^{12}C}$ and $I_{550,^{12}C}$ correspond to the peak height of the tracings at m/e 548 and 550, respectively, in the MID recording of the derivative of unlabeled corticosterone. $I_{548,^{14}C}$ and $I_{550,^{14}C}$ correspond to the peak height of the tracings at m/e 548 and 550, respectively, in the MID recording of the derivative of 4-¹⁴C-labeled corticosterone.

The same equation was used for calculation of endogenous corticosterone present in the mitochondrial fraction prior to incubation; in this case P_{ex} corresponded to the amount of [4-¹⁴C]corticosterone added before extraction. Labeled and unlabeled aldosterone were calculated according to the same principle. Due to lack of 18-hydroxycorticosterone of sufficiently high specific radioactivity (synthesis from [4-¹⁴C]corticosterone proved futile), the amounts of labeled and unlabeled 18-hydroxycorticosterone were calculated using corticosterone as labeled standard (cf. below). It is then assumed that the ratio $I_{548,^{14}C}/I_{550,^{14}C}$ for labeled corticosterone is the same as the ratio $I_{605,^{14}C}/I_{607,^{14}C}$ for labeled 18-hydroxycorticosterone. It should be pointed out that the ratio between the tracings at m/e 605 and 607, corresponding to unlabeled 18-hydroxycorticosterone, was exactly the same as the ratio between m/e 548 and 550, corresponding to unlabeled corticosterone.

RESULTS

Mass fragmentographic assay

Fig. 1 shows typical mass fragmentographic recordings of the ions at m/e 548, 550 (corticosterone), 591, 593 (aldosterone), 605, and 607 (18-hydrocorticosterone) of a mixture of MO-TMS derivatives of unlabeled corticosterone, 18-hydroxycorticosterone, and aldosterone. Fig. 2 shows mass fragmentographic recordings of the corresponding ions of [4-¹⁴C]cortico-

sterone and [4-¹⁴C]aldosterone. Evidently, the radioactive materials contained about 90% ¹⁴C-labeled molecules. Due to lack of labeled 18-hydroxycorticosterone of sufficiently high specific radioactivity (cf. Methods), no recordings of this labeled compound can be shown.

Fig. 3 shows the mass fragmentographic recordings of six ions of an extract of an incubation of [4-¹⁴C]corticosterone with the mitochondrial fraction. No interference by contaminating compounds in the mass fragmentographic recordings of the ions specific for

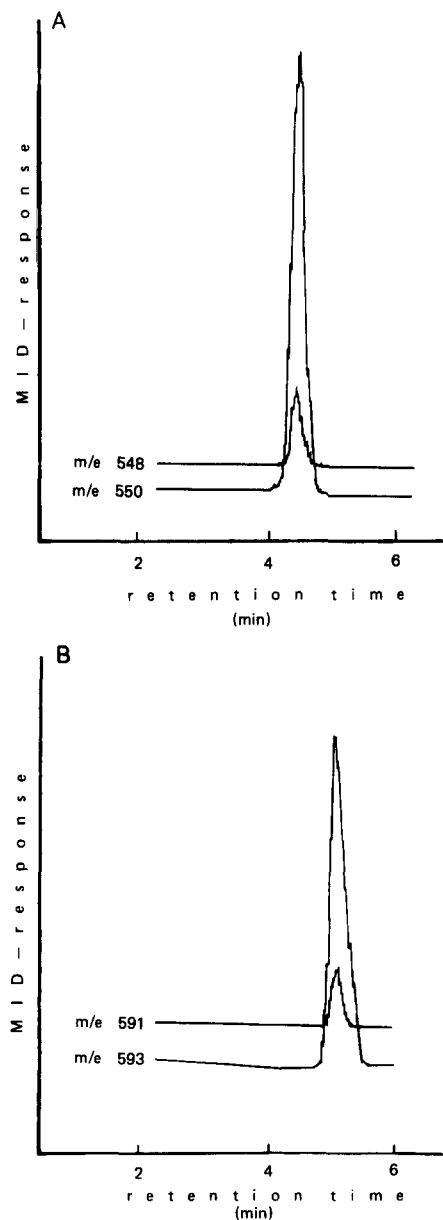


Fig. 2. MID recordings of labeled MO-TMS derivatives. A shows the MID recording of the MO-TMS derivative of [4-¹⁴C]corticosterone (m/e 548 and 550). B shows the MID recording of [4-¹⁴C]aldosterone at m/e 591 and 593. For further details, see Methods.

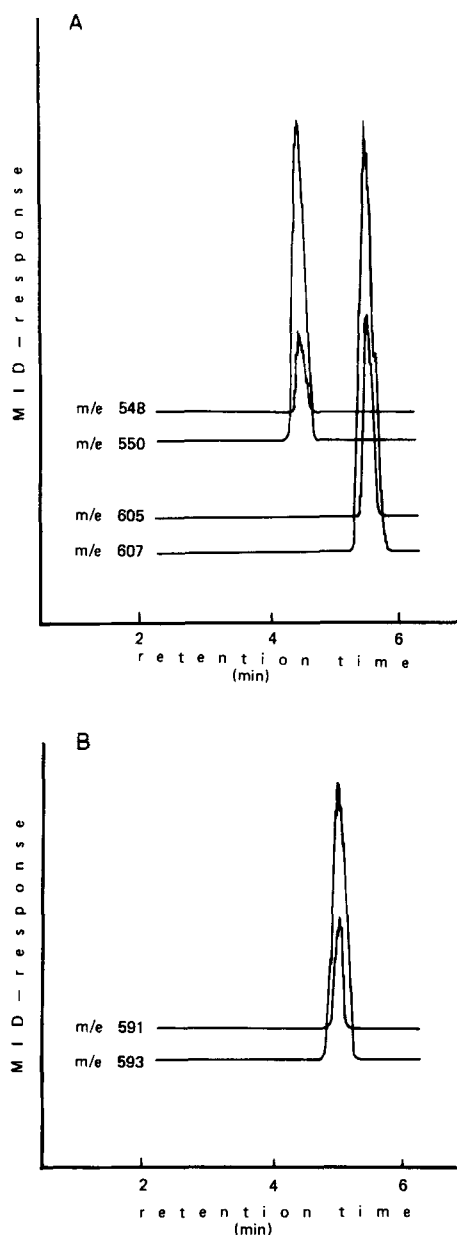


Fig. 3. MID recordings of the methylene chloride extract of an incubation of [4-¹⁴C]corticosterone with the mitochondrial fraction. Standard assay conditions were used. For experimental details, see Methods and Figs. 1 and 2.

18-hydroxycorticosterone, aldosterone, or corticosterone could be detected. Due to unspecific adsorption on the column, at least 2–4 ng of each steroid had to be injected into the gas chromatograph–mass spectrometer in each analysis to give satisfactory tracings for the respective steroid. Above 4 ng neither the amount of steroid injected nor the sample volume influenced the ratio obtained between labeled and unlabeled molecules in the MID analysis.

It was shown that 18-hydroxycorticosterone and aldosterone originated from corticosterone and were

not products of 18-hydroxydeoxycorticosterone. Thus, incubation of [4-¹⁴C]18-hydroxydeoxycorticosterone (235 ng, isotopic abundance 46%) under standard assay conditions resulted in <0.5% formation of labeled corticosterone, 18-hydroxycorticosterone, and aldosterone, respectively, when assayed by radioscanning of the thin-layer plate or radio gas-liquid chromatography. Assay by mass fragmentography as above showed that only unlabeled material corresponding to 18-hydroxycorticosterone and aldosterone could be found in the incubation extract. The latter products must be derived from unlabeled corticosterone. Incubation of 235 ng of [4-¹⁴C]corticosterone under equivalent conditions resulted in a conversion of >15% into labeled 18-hydroxycorticosterone and aldosterone. The amount of unlabeled material formed after incubation with the small amount of [4-¹⁴C]corticosterone and [4-¹⁴C]18-hydroxydeoxycorticosterone, respectively, was approximately the same as that obtained in the incubations with saturating amounts of [4-¹⁴C]corticosterone (cf. below).

In most incubations conversion into aldosterone was low or insignificant. If significant amounts of aldosterone were formed, the ratio between labeled and unlabeled aldosterone was identical to that between labeled and unlabeled 18-hydroxycorticosterone. If radioactivity with the chromatographic properties of aldosterone was absent in an incubation, no aldosterone could be detected by mass fragmentography.

The amount of endogenous corticosterone present prior to incubation was determined by mass fragmentography. Mitochondria and cytochrome P-450 (fortified with NADPH-cytochrome P-450 reductase or adrenodoxin/adrenodoxin reductase) used in the standard assay procedures contained less than 30 ng and 10 ng, respectively, of unlabeled corticosterone. After incubation the content of unlabeled corticosterone increased six-fold (mitochondria) or remained constant (reconstituted systems). No significant amount of endogenous aldosterone (<3 ng) or 18-hydroxydeoxycorticosterone (<0.05 ng) could be detected prior to incubation.

The validity of the method was confirmed by mass fragmentographic analysis of incubation mixtures containing a known amount of labeled corticosterone and different amounts of added unlabeled corticosterone. There was good agreement between expected and found values and the maximal error was 4.5% (Table 1). Table 2 shows results of experiments in which known amounts of unlabeled 18-hydroxycorticosterone had been added to incubated incubation mixtures. In this case, also, there was good agreement between calculated and found values and the maximal difference was 5.5%.

TABLE 1. Recovery of corticosterone added to incubated mitochondrial preparations

	Unlabeled Corticosterone		Difference Found/Calculated
	μg	%	
Mitochondrial fraction before incubation	0.01 ^a		
Incubated mitochondrial fraction	0.06 ^b		
Mitochondrial fraction after incubation + 2 μg of corticosterone	2.01	2.4	
Mitochondrial fraction after incubation + 5 μg of corticosterone	5.08	0.3	
Mitochondrial fraction after incubation + 10 μg of corticosterone	10.45	3.9	
Mitochondrial fraction after incubation + 15 μg of corticosterone	15.72	4.4	
Mitochondrial fraction after incubation + 20 μg of corticosterone	20.84	3.9	

Standard assay conditions were used. Different amounts of unlabeled corticosterone were added prior to extraction and assay by mass fragmentography (cf. Methods).

^a Mean value from nine experiments. SEM = 0.004 μg .

^b Mean value from nine experiments. SEM = 0.02 μg .

Assay conditions for 18-hydroxylation of endogenous and exogenous corticosterone by rat adrenal mitochondria

Fig. 4 summarizes experiments in which 18-hydroxylation of endogenous and exogenous corticosterone was assayed in mitochondria with the mass fragmentographic technique. Under the conditions employed, 18-hydroxylation was linear with time for up to 40 min and with mitochondrial protein up to 0.8 mg. The enzyme was saturated with 5 μg of exogenous steroid. At substrate saturation level, the ratio between 18-

hydroxycorticosterone formed from labeled and unlabeled corticosterone was about constant (3:2). The addition of [4-¹⁴C]corticosterone did not significantly influence formation of endogenous 18-hydroxycorticosterone, indicating that labeled and unlabeled corticosterone were not in equilibrium.

Assay conditions for 18-hydroxylation of exogenous and endogenous corticosterone by reconstituted systems

Table 3 shows experiments in which the conversion of endogenous and 4-¹⁴C-labeled corticosterone was

TABLE 2. Recovery of 18-hydroxycorticosterone added to incubated mitochondrial preparations

	18-Hydroxycorticosterone		Difference Found/Calculated
	¹⁴ C	¹² C	
	ng		%
Mitochondrial fraction before incubation	0.0	3.0	
Incubated mitochondrial fraction	125.1 ^a	73.8 ^b	
Mitochondrial fraction after incubation + 10 ng of 18-hydroxycorticosterone	130.3	79.2	5.5
Mitochondrial fraction after incubation + 20 ng of 18-hydroxycorticosterone	122.7	93.5	0.3
Mitochondrial fraction after incubation + 50 ng of 18-hydroxycorticosterone	127.4	118.5	4.3
Mitochondrial fraction after incubation + 100 ng of 18-hydroxycorticosterone	126.6	169.4	2.5

Standard assay conditions were used. Different amounts of unlabeled 18-hydroxycorticosterone (quantitated by UV absorption at 241 nm (22) with corticosterone as standard) were added prior to extraction and assay by mass fragmentography (cf. Methods).

^a Mean value obtained from nine different parallel experiments. SEM = 3.4 ng.

^b Mean value obtained from nine different parallel experiments. SEM = 3.0 ng.

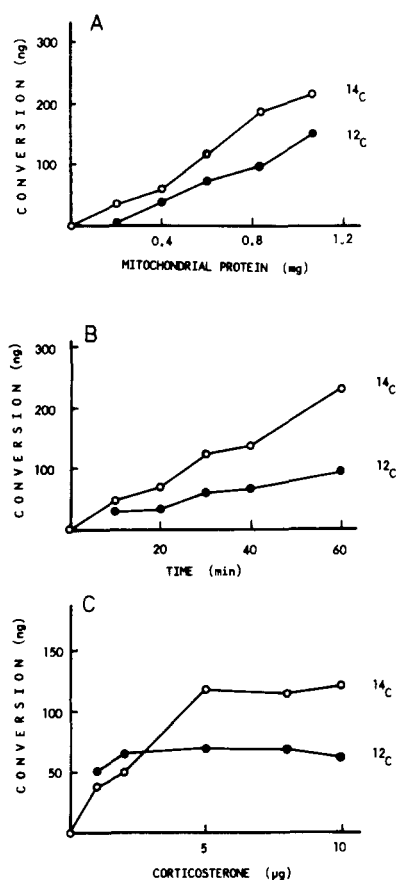


Fig. 4. Effect of enzyme concentration (A), time (B), and substrate concentration (C) on 18-hydroxylation of exogenous ^{14}C -labeled and endogenous unlabeled corticosterone by the mitochondrial fraction of a rat adrenal homogenate. Standard assay conditions were used. For experimental details, see Methods. ● — ●, 18-Hydroxylation of unlabeled corticosterone; ○ — ○, 18 hydroxylation of labeled corticosterone.

assayed in a reconstituted system containing rat adrenal cytochrome P-450 (0.3–0.4 nmol) and bovine NADPH-cytochrome P-450 reductase (40 units). In the presence of nonlimiting amounts of NADPH-cytochrome P-450 reductase (40 units) 18-hydroxylation of labeled corticosterone was linear with time for at least 60 min and with cytochrome P-450 up to about 0.5 nmoles. The enzyme was saturated with about 4 μg of [4- ^{14}C]corticosterone. Regardless of the amount of [4- ^{14}C]corticosterone added, formation of unlabeled 18-hydroxycorticosterone was about constant. The ratio between labeled and unlabeled 18-hydroxycorticosterone at substrate saturation was about 10:1.

Effect of treatment with potassium chloride on catalytic activity of rat adrenal mitochondria

Table 4 summarizes results of experiments with adrenal mitochondria from groups of rats given 0.15–0.45 mol/l potassium chloride in drinking fluid. The

amount of adrenal cytochrome P-450 increased with increasing concentrations of potassium chloride in drinking fluid and reached a level about 2-fold higher than that of control animals. 18-Hydroxylation of exogenous corticosterone increased concomitant to the increase in amount of cytochrome P-450 and reached a level about twice that of control animals. 18-Hydroxylation of endogenous corticosterone, however, was increased only to a low and insignificant degree by the treatment. 18-Hydroxylation of deoxycorticosterone also increased with increasing concentration of potassium chloride and reached a level about 2-fold higher than that obtained in control animals. The stimulatory effect of the lowest concentration of potassium chloride in drinking fluid on 18-hydroxylation of deoxycorticosterone (about 25%) was less than that of exogenous corticosterone (about 100%). 11 β -Hydroxylation of deoxycorticosterone was stimulated to the same degree as 18-hydroxylation of the same substrate (cf. 5).

Fig. 5 summarizes results of experiments in which rats were treated with 0.3 mol/l potassium chloride for different periods of time. 18-Hydroxylation of exogenous corticosterone was stimulated earlier than 18-hydroxylation and 11 β -hydroxylation of deoxycorticosterone. Thus maximal 18-hydroxylation of exogenous corticosterone occurred already after treatment with potassium chloride for 6 hr, whereas maximal 18-hydroxylation and 11 β -hydroxylation of deoxycorticosterone occurred first after treatment for 4 days. It should be pointed out that the amount of cytochrome P-450 was not significantly increased during the first day of treatment.

TABLE 3. Hydroxylation of labeled and unlabeled corticosterone by rat cytochrome P-450

	18-Hydroxycorticosterone	
	^{12}C	^{14}C
	ng/30 min	
Rat cytochrome P-450	<3	
Bovine NADPH-cytochrome P-450 reductase	<3	
Rat cytochrome P-450 + NADPH-cytochrome P-450 reductase	13	155
Adrenodoxin	<3	
Adrenodoxin reductase	<3	
Rat cytochrome P-450 + adrenodoxin	<3	
Rat cytochrome P-450 + adrenodoxin reductase	<3	
Rat cytochrome P-450 + adrenodoxin + adrenodoxin reductase	7	82

In a reconstituted system cytochrome P-450 (0.36 nmol) was incubated with bovine NADPH-cytochrome P-450 reductase (40 units) or purified fractions of adrenodoxin (0.05 mg) and adrenodoxin reductase (1.0 mg) corresponding to 20 units of cytochrome c reductase activity.

TABLE 4. Effect of treatment with potassium chloride on 18-hydroxylation of corticosterone and deoxycorticosterone and 11 β -hydroxylation of deoxycorticosterone in rat mitochondrial fraction

Potassium Chloride Concentration	Adrenal Cytochrome P-450	18-Hydroxylation of Corticosterone		18-Hydroxylation of Deoxycorticosterone	11 β -Hydroxylation of Deoxycorticosterone
		¹² C	¹⁴ C		
<i>mol/l</i>	<i>nmol</i>	<i>nmol converted \times (mg of protein)⁻¹ \times min⁻¹</i>			
0.00	0.6	0.014	0.018	1.23	3.88
0.15	0.8	0.017	0.038	1.67	4.81
0.30	1.5	0.012	0.037	2.07	6.32
0.45	1.4	0.015	0.034	2.22	6.84

Standard assay conditions were used. The figures are the mean of results from two rats. Analytical variation is given in experimental section.

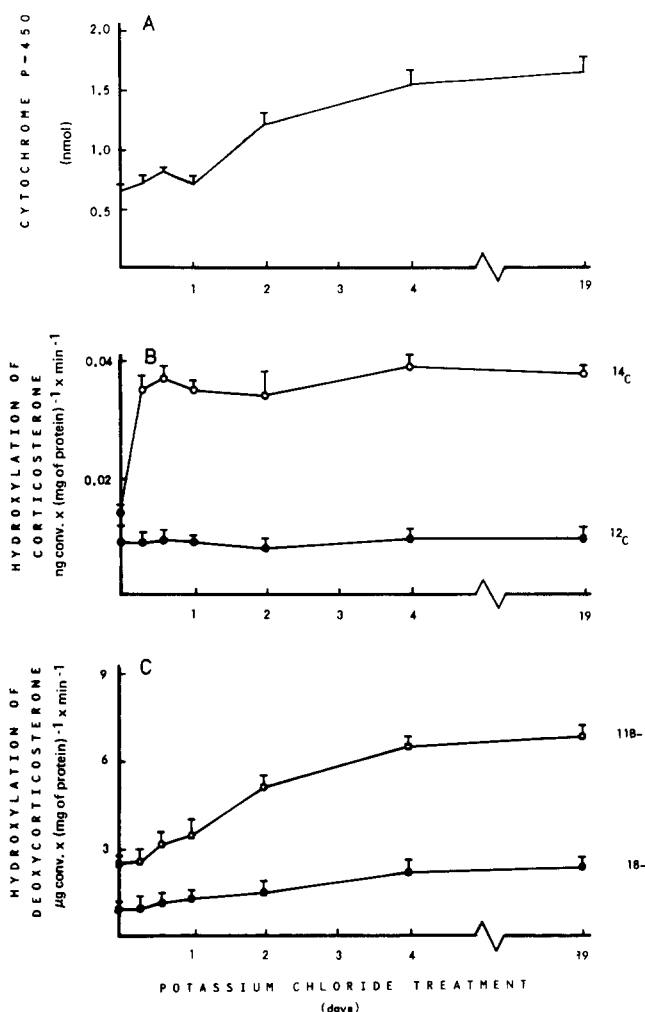


Fig. 5. Effect of potassium chloride treatment (0.3 mol/l) on 18-hydroxylation of endogenous and exogenous corticosterone and 18- and 11 β -hydroxylation of deoxycorticosterone for different periods of time. Standard assay conditions were used. The figures given are the mean and SEM of results from six rats. Three independent experiments with two rats in each experiment were performed. For further details, see Methods. ●—●, 18-Hydroxylation of unlabeled corticosterone; ○—○, 18-hydroxylation of labeled corticosterone; ■—■, 18-hydroxylation of labeled deoxycorticosterone; □—□, 11 β -hydroxylation of labeled deoxycorticosterone; —, total content of mitochondrial cytochrome P-450.

Effect of canrenone, canrenoate-K, and spironolactone on catalytic activity of rat adrenal mitochondria

Fig. 6 summarizes results of experiments in which canrenone, canrenoate-K, and spironolactone were used as inhibitors of 18-hydroxylation of labeled and unlabeled corticosterone and 18- and 11 β -hydroxylation of [1,2-³H₂]deoxycorticosterone. For comparative reasons, both substrates were incubated in Krebs-Ringer buffer. Addition of an amount of canrenoate-K equivalent to a ratio between inhibitor and exogenous substrate of about 2 (Fig. 6B) resulted in a decrease in hydroxylase activity of both labeled and unlabeled corticosterone of about 50%. The corresponding ratios for canrenone and spironolactone were 4 and 10, respectively, indicating that the latter inhibitors were less potent than canrenoate-K. In all experiments, hydroxylation of endogenous corticosterone was influenced in a similar way as hydroxylation of [4-¹⁴C]-corticosterone. The inhibitors were less effective in incubations with deoxycorticosterone as substrate. Thus, in order to give about 50% inhibition of 11 β - and 18-hydroxylation of deoxycorticosterone, canrenone, canrenoate-K, and spironolactone had to be added in greater excess. An inhibitory effect of about 50% was found if the incubations contained 12–14 (canrenone) or 15–18 (canrenoate-K and spironolactone) times more inhibitor than the constant amount of substrate.

DISCUSSION

Assay conditions and properties of 18-hydroxylation of exogenous and endogenous corticosterone

The present mass fragmentographic technique to assay 18-hydroxylation of endogenous and exogenous corticosterone in adrenal preparations is relatively simple and seems to be accurate in the appropriate range. The labeled substrate and products serve as

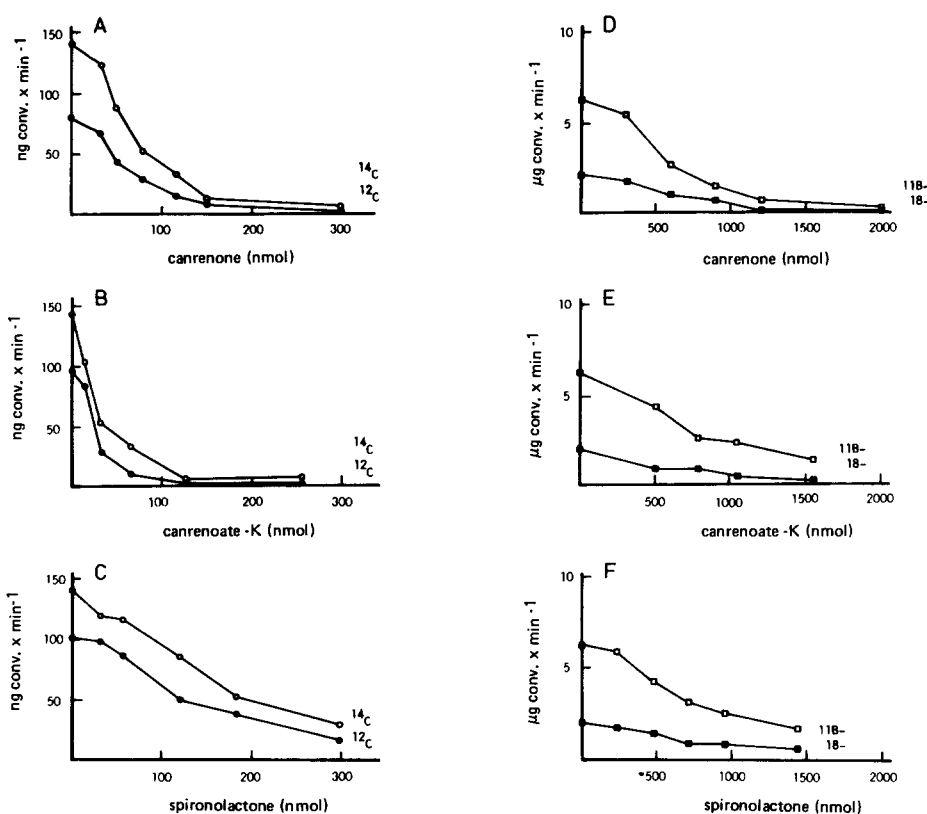


Fig. 6. Effect of canrenone, canrenoate-K, and spironolactone on 18-hydroxylation of endogenous and exogenous corticosterone (A–C) and 18- and 11 β -hydroxylation of deoxycorticosterone (D–F). Suboptimal amounts of [4- 14 C]corticosterone (2 μ g) and [1,2- 3 H $_2$]deoxycorticosterone (15 μ g) were used. Canrenone, canrenoate-K, and spironolactone were added before preincubation (see Methods). Rate of hydroxylation is expressed as nmol converted substrate \times min $^{-1}$. \bullet — \bullet , 18-Hydroxylation of unlabeled corticosterone; \circ — \circ , 18-hydroxylation of labeled corticosterone; \blacksquare — \blacksquare , 11 β -hydroxylation of labeled deoxycorticosterone; \square — \square , 11 β -hydroxylation of unlabeled deoxycorticosterone.

ideal internal standards in the procedure and no corrections for losses in the extractions or in the chromatography are necessary. The derivatives used have relatively high molecular weight, are stable, and give one single peak under the gas-liquid chromatographic conditions employed (17, 18). The specificity of the method should be high in view of the small probability that contaminating compounds both have the same gas-liquid chromatographic properties and contain the same specific ions in their mass spectra as the compound to be assayed. Above a certain amount of steroid, the assay was not influenced by injection of various volumes or different amounts of steroid into the gas chromatograph-mass spectrometer.

18-Hydroxylation of exogenous corticosterone by rat adrenal mitochondria and reconstituted systems was found to be linear with enzyme concentration and time. In reconstituted systems it was shown that the rate of hydroxylation was determined only by the cyto-

chrome P-450 fraction, provided that NADPH-cytochrome P-450 reductase was added in excess. In similarity to 18-hydroxylation of deoxycorticosterone (4), both components constituting the NADPH-cytochrome P-450 reductase activity, adrenodoxin and adrenodoxin reductase, had to be present. It may be concluded that the mechanism for electron transport is the same in the two hydroxylations.

Under optimal assay conditions, and after addition of saturating amounts of [4- 14 C]corticosterone to the mitochondrial fraction, the ratio between the formation of 18-hydroxycorticosterone from labeled and unlabeled corticosterone was about 3:2. This ratio was influenced only to a very small degree by the addition of different amounts of labeled corticosterone, indicating that endogenous corticosterone did not equilibrate with exogenous steroid. A similar lack of equilibrium between endogenous steroid and exogenously added steroid has been observed in connection with studies on side-chain cleavage of cholesterol in adrenal

mitochondrial preparations (10). In reconstituted systems the rate of conversion of endogenous corticosterone was relatively small. This was probably due to the fact that the amount of endogenous steroid was low prior to incubation and that no endogenous corticosterone could be formed from precursors during incubation of these partially purified fractions.


The most efficient 18-hydroxylation of endogenous and exogenous corticosterone by rat adrenal mitochondria was obtained with isocitrate as the source of reducing equivalents. In contrast, 18-hydroxylation of deoxycorticosterone is more efficient with NADPH, provided that Ca^{2+} or K^{+} is present in the buffer for swelling of the mitochondria (4). When assayed in the presence of NADPH and K^{+} , the rate of 18-hydroxylation of corticosterone was low and it was not possible to obtain optimal assay conditions with respect to time and concentration of enzyme. In addition, results were much less reproducible under these conditions.

Heterogeneity of the 18-hydroxylating system

In a previous study from this laboratory, the possibility was discussed that 18-hydroxylation of deoxycorticosterone and of corticosterone may be catalyzed by different enzyme systems (5). This contention is substantiated by some of the results of the present work. Although treatment with potassium chloride stimulated 18-hydroxylation of both substrates (5), the pattern of induction was different. 18-Hydroxylation of deoxycorticosterone was stimulated gradually during the first 4 days, and the increase in activity closely followed the increase in concentration of cytochrome P-450. 18-Hydroxylation of corticosterone, however, was already maximally stimulated after 6–12 hr. This high activity was obtained without significant change in the concentration of cytochrome P-450. Thus it seems probable that the cytochrome P-450 involved in 18-hydroxylation of corticosterone is different from the bulk of cytochrome P-450 in adrenal mitochondria.

Further evidence for heterogeneity of the 18-hydroxylating system was obtained from the inhibition experiments. In consonance with the work by Cheng et al. (11), 18-hydroxylation of labeled corticosterone was inhibited by the addition of canrenone, canrenoate-K, or spironolactone. The inhibition of hydroxylation of endogenous substrate was of similar magnitude as that of exogenously added corticosterone. 18-Hydroxylation of deoxycorticosterone was reduced to a smaller extent by each inhibitor than was 18-hydroxylation of corticosterone. These experiments support the contention that different binding sites and possibly different species of cytochrome P-450 may be involved in the 18-hydroxylation of corticosterone and deoxycorticosterone.

It was shown in previous work that 18- and 11β -hydroxylation of deoxycorticosterone were closely associated under a variety of different experimental conditions and it has been suggested that the two activities are catalyzed by the same enzyme system (4, 5, 19). In accordance with this contention, in the present work the two activities followed each other during the different experimental conditions (treatment with potassium chloride and addition of different inhibitors *in vitro*). It may be mentioned that Cheng et al. (11) found a difference between 18-hydroxylation of corticosterone and 11β -hydroxylation of deoxycorticosterone in their inhibition experiments, similar to that found in the present work.

It has been suggested that 18-hydroxylation of endogenous and exogenous corticosterone may be catalyzed by different mechanisms (7, 8). The finding that treatment with potassium chloride stimulated 18-hydroxylation of exogenous corticosterone considerably more than that of endogenous corticosterone may at first sight support such a hypothesis. The explanation may be, however, that only part of the endogenous steroid is available for hydroxylation. A similar situation has been found in studies on side-chain cleavage of endogenous and exogenous cholesterol in rat adrenals (6, 10). It should also be pointed out that some "endogenous" corticosterone may be lost during the preparation of the mitochondrial fraction. It has been shown that after homogenization and centrifugation about 60% of the corticosterone present in an adrenal gland can be recovered in the soluble fraction (20). Thus the bulk of the endogenous corticosterone present in the adrenal gland is not available as substrate for the enzyme system in the isolated mitochondrial fraction. Under *in vivo* conditions, this bulk of endogenous corticosterone may be hydroxylated in a similar way as the added labeled corticosterone is hydroxylated under *in vivo* conditions. The possibility must be considered that the labeled and unlabeled products obtained *in vitro* are derived from different substrates. It was clearly shown, however, that 18-hydroxydeoxycorticosterone hardly could be a precursor to the unlabeled 18-hydroxycorticosterone obtained as product. Thus the mitochondrial fraction was practically devoid of unlabeled 18-hydroxydeoxycorticosterone and a trace amount of unlabeled 18-hydroxydeoxycorticosterone was not converted into 18-hydroxycorticosterone to a measurable extent. The contention that 18-hydroxydeoxycorticosterone is of little or no importance as an intermediate in the formation of 18-hydroxycorticosterone and aldosterone is in accordance with previous studies (2, 8, 21).

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