18-SUBSTITUTED STEROIDS. PART 15. 6β-HYDROXYLATION OF ALDOSTERONE BY LIVER

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ABSTRACT

6β-Hydroxyaldosterone and 6β-hydroxy-17-isoaldosterone, characterized by high-field NMR studies, are among the major polar metabolites formed from aldosterone by incubation with rat liver slices or microsomal fraction. It is uncertain at present whether the 17-iso product results from an enzymatic or a chemical inversion of configuration. Periodate degradation of the 6β-hydroxyaldosterone gave 6β-hydroxyaldosterone γ-lactone, identical with a synthetic sample.

INTRODUCTION

Significant quantities of Ring-A-reduced and other polar neutral metabolites of aldosterone (NMA) are present in liver and kidney of male rats during the latent period of this hormone (1,2). Experiments in these laboratories (by S.A.L. and D.J.M.) have suggested that some of the metabolites may be physiologically important in the regulation and/or expression of the hormone's action in the kidney (2). The polar NMA in vivo in liver and kidney have now been resolved by high pressure liquid chromatography (HPLC) into at least 20 major peaks of metabolites eluting in 4 regions of increasing

polarity, designated A, B, C, and D (2) (Fig. 1). Resolution of these metabolites has recently been improved by using gradients of aqueous methanol or aqueous acetonitrile and by employing heated, jacketed reverse-phase HPLC columns. Detection of the radiolabeled metabolites of aldosterone can now be achieved with on-line β-radioactivity counters (2). Several of the polar NMA can be synthesized by microsomal cytochrome P450-dependent enzymes isolated from rat liver (3). The polar NMA are the major products when low concentrations (< 10 μM) of aldosterone are used. We are in the process of isolating sufficient quantities of each of them for structure determination using nuclear magnetic resonance (NMR) and mass spectral studies. This paper describes the NMR analysis of those polar NMA synthesized by in vitro preparations which comprise the major peak eluting in the B region. At the concentration of aldosterone used in this experiment (185 μM), 25% of the total radioactivity was recovered in the NMA fractions, with 10% of that total being in the peak which is the subject of the present paper. Significant quantities of metabolites which chromatograph in both the A and C regions are also formed in vitro. A description of their synthesis, isolation, and chemical characterization will be published elsewhere.

MATERIALS AND METHODS

Chemicals

[1,2-^{3}H]Aldosterone with a specific activity of 46.2 Ci/mmol was obtained from New England Nuclear Corporation (Boston, Mass.). The [1,2-^{3}H]aldosterone was purified by HPLC before use. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADPH, Trizma base (tris-hydroxymethylamino-methane) and $MgCl_2$ were obtained from Sigma Chemical Co. Non-radioactive aldosterone was obtained from Sigma Chemical Co. or Andard-Mount (London) Ltd. (Wembley, Middx., U.K. 'Light petroleum' refers to the fraction of bp 60-80 C.

Animals

Intact adult male Sprague-Dawley rats (Caesarian derived, from Charles River, Wilmington, MA) 50-70 days old were used in

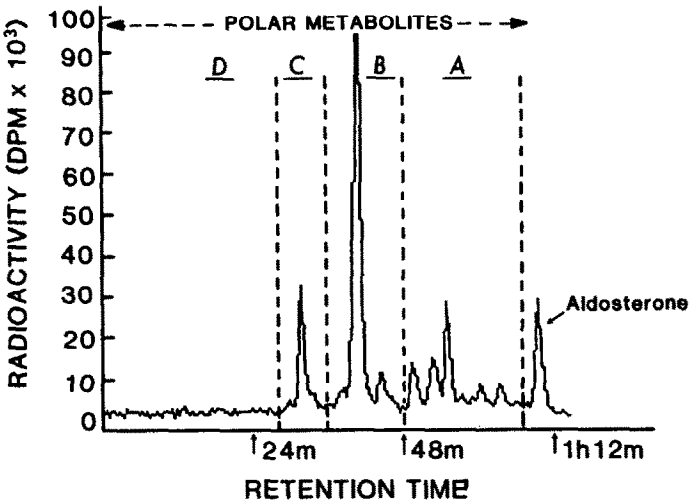


Figure 1. HPLC of the extract from male rat liver microsomal incubations.

all experiments. The animals were fed Purina Laboratory chow and tap water ad libitum. Phenobarbital, a known inducer of liver microsomal enzymes, was injected IP (100 mg/kg body weight) for 5 days. On the fifth day, the rats were bilaterally adrenalectomized 24 h prior to sacrifice. Adrenalectomized rats were maintained on 0.9% saline drinking water.

Preparation of Microsomal Fraction

Rat liver was homogenized in 0.25 M sucrose (5:1, vol:wt) using a Teflon homogenizer. The microsomal fraction was obtained by differential centrifugation using a Sorvall RC-2 preparative centrifuge and a Beckman L8-80 ultracentrifuge. All centrifugation steps were performed at 4 C. The homogenate was centrifuged at 1,000 x g for 10 min, and the supernatant was recentrifuged at 9,000 x g and then at 17,000 x g for 10 min each time. The 17,000 x g supernatant was centrifuged at 105,000 x g (70 min). The resultant pellet (microsomal fraction) was rinsed with 0.25 M sucrose and suspended in 0.25 M sucrose at a concentration of 30 mg protein/mL. Protein concentration was determined by the Biuret method (4).

In Vitro Incubation

Large scale synthesis of polar aldosterone metabolites eluting between 36 and 42 min

Microsomal (105,000 x g) fraction was incubated with 185 μM aldosterone (20 mg; together with 250 μCi [^{3}H]aldosterone) dissolved in 1% ethanolic 50 mM Tris-HCl buffer, pH 7.4, containing 500 μM NADPH, 50 mM glucose-6-phosphate, 1,000 units of glucose-6-phosphate dehydrogenase and 5 mM $MgCl_2$ in a reaction volume of 300 mL. The reaction mixture was bubbled with O_2 and incubated at 37 C with constant shaking for 1 h and then cooled in ice for 15 min to terminate reaction. Aldosterone and its metabolites were then extracted with Sep-pak C_{18} cartridges (Waters Associates, Milford, MA) according to the method of Morris and Tsai (5). Cartridges were first prepared by washing with MeOH (5 mL) and then with H_2O (5 mL). The microsomal incubation mixture was extracted by passing through the Sep-pak cartridge, followed by washing with H_2O (5 mL) and elution with MeOH (5 mL). As a control experiment, a sample of aldosterone was subjected to the full sequence of steps involved in its incubation in buffered medium and the subsequent extraction and separation of metabolites, but omitting only the liver microsomal fraction.

High Pressure Liquid Chromatography

The extract was purified on a C_8 Zorbax (DuPont) column and metabolites of aldosterone were separated using a stepwise gradient of aqueous methanol (15% MeOH for 20 min, 35% MeOH for 30 min, and 50% MeOH for 35 min) at a flow rate of 1 mL/min at room temperature. The eluate was collected as 1 mL fractions in siliconized vials using a fraction collector. A total of 8-10 injections was made to separate all of the incubation extract. An aliquot from each vial was mixed with 5 mL Instagel liquid scintillation fluid (Packard Instruments, Downers Grove, IL) and counted for ^{3}H-radioactivity.

Metabolites eluting in the 36-42 min fractions were collected and processed for the NMR studies reported in this paper (see below). Other metabolite fractions eluting between 30 and 33 min, 43 and 46 min, 48 and 51 min, 52 and 54 min, 55 and 56 min, and 59 and 62 min, were saved for further structural determinations. Ring-A-reduced metabolites of lower polarity, also synthesized under these conditions, were collected from fractions eluting after 62 min.

For further purification, the combined 36-42 min fractions were rechromatographed on a Spherisorb (10 μ) column with UV detection at 240 nm, with a second solvent system employing aqueous 40% methanol as mobile phase at room temperature at a flow rate of 2 mL/min. UV-absorbing fractions were taken to dryness under reduced pressure, and the residues were dissolved in CD_3OD for ^1H NMR spectroscopy.

NMR Spectroscopy

NMR spectra were determined at 400 MHz on a Bruker WH-400 spectrometer (University of London Intercollegiate Research Service, Queen Mary College), or at 500 MHz on a Bruker AM-500 spectrometer (National Institute for Medical Research, Mill Hill, London). Unless otherwise stated, the solvent was methanol-d_4 ('ultra puriss' grade: >99.95 atom % ^2H) from Ciba-Geigy.

Two-dimensional ^1H-homonuclear shift-correlated spectra (COSY-45) (6) were obtained with 2048 data points in the f_2 dimension and zero-filling to 1024 data points in the f_1 dimension (512 or fewer actual experiments were usually acquired) to achieve a symmetrical data matrix on transformation. A shifted sine-bell window function was used.

Infrared spectra were determined for KBr discs on a Mattson Polaris FT-IR instrument.

Synthesis of 11β,18-epoxy-6β-hydroxy-3-oxoandrost-4-ene-17β,18-carbolactone ('6β-hydroxyaldosterone-γ-lactone')

11β,18-Epoxy-3-oxoandrost-4-ene-17β,18-carbolactone ['aldosterone γ-lactone', prepared by oxidizing aldosterone with $NaIO_4$ (7)] (84 mg) was treated with a solution of perchloric acid (60%, 10 μL) and acetic anhydride (4.8 mL) in ethyl acetate (45 mL) and stirred. After 30 min the lactone had dissolved to give a pale yellow solution. Aqueous sodium hydrogen carbonate (100 mL, saturated) was added and the mixture was stirred for 30 min, then the ethyl acetate layer was separated, washed with water, dried (Na_2SO_4), and the solvent was removed under reduced pressure to give the crude enol acetate, which crystallized from aqueous acetone as needles (67.3 mg) mp 248-260 C; ν_{max} 1774 (lactone C=O), 1741 (acetate), 1660 and 1627 (diene), 1224, 1117, and 895 cm^{-1}.

TLC on Kieselgel 60 F_{254} plates (Merck) with ethyl acetate/light petroleum (60:40) showed a single spot, R_f 0.66 (cf. the original γ-lactone, R_f 0.24).

The crude enol acetate (35 mg) in dioxan (10 mL) was treated with iodosobenzene [50 mg, freshly prepared by stirring finely powdered iodobenzene diacetate (1 g) with sodium hydroxide solution (2.0 M, 5 mL) for 1 h, and filtering] followed by aqueous sodium hydroxide (0.1%, 5 mL). The mixture was stirred for 12 h, when the yellow iodosobenzene had been replaced by a colorless precipitate. Dichloromethane (30 mL) and water (30 mL) were added and the mixture was stirred, then the dichloromethane layer was separated, washed with water (2 x 50 mL), and taken to dryness under reduced pressure.

Flash chromatography of the product on a column of Kieselgel $PF_{254+366}$ (Merck), eluting with ethyl acetate:light petroleum (60:40), gave fractions containing traces of aldosterone γ-lactone, followed by 6β-hydroxyaldosterone γ-lactone (18 mg) and a mixture the 6β- and 6α-hydroxy derivatives which was not separated further. 6β-Hydroxy-aldosterone γ-lactone formed prisms from acetone/hexane, mp 266-272 C (decomp.); ν_{max} 3350 (OH), 1775 (lactone C=O), 1662 (3-C=O), 981, and 890 cm^{-1}. NMR spectrum - see Table 1.

RESULTS

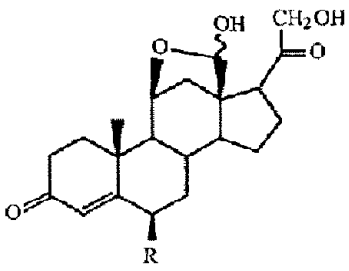
Two main UV-absorbing peaks from rechromatography of the 36-42 min material (Fig. 1) were eluted at 2.7 min and 5.0 min, respectively. These UV-absorbing fractions, each approximately 0.5 mg, were dissolved in CD_3OD for ^1H NMR spectroscopy. Other elutes were essentially UV-transparent, and contained only minute traces of material which were rejected.

Interpretations of the NMR spectra were greatly aided by our recent full analysis of the ^1H spectrum of aldosterone itself (8). Aldosterone exists in solution as an equilibrating mixture of 20-oxo 1 and 18,20-hemiacetal 2 tautomers; the ^1H NMR spectrum shows the features of both structures, in a ratio approximating to 3:4, respectively. For the present study

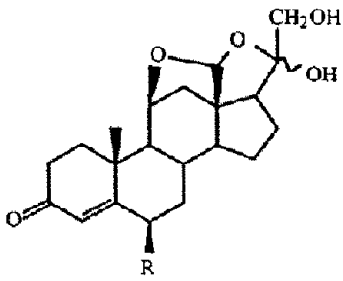
Table 1. ^{1}H NMR spectra of 6β-hydroxylated aldosterone derivatives and reference compounds[a]

H	6β-OH-Prog[b,c]	ALDO[d]	6β-OH-ALDO[e] (2.7min fraction)	17-iso-ALDO 3 (5.0min fraction)	6β-OH-17-iso-ALDO 8	Lactone 5[c]	6β-OH-lactone 7[c]
1α	1.72	1.74	1.73	1.69	1.71	1.73	1.76
1β	2.05	2.22	2.16	2.17	2.16	2.22	2.21
2α	2.40	2.36	2.34	2.43	2.33	2.38	2.35
2β	2.53	2.55	2.64	2.55	2.62	2.53	2.64
4	5.83	5.71	5.78	5.70	5.78	5.74	5.79
6α	4.36	2.36	4.28	2.44	4.27	2.32	4.27[g]
6β	-	2.55	-	2.52	-	2.44	-
7α	1.28	1.26	[f]	1.18	1.41	1.29	1.53[h]
7β	2.02	2.06	2.12	2.02	ca 2.13	2.03	2.09[i]
8	1.99	1.82	[f]	1.76	ca 2.13	ca 1.7	2.19
9	0.97	1.15	1.14	0.98	0.97	1.16	1.25
11α	1.63	4.81	4.79	4.52	4.52	4.91	4.90
11β	1.53	-	-	-	-	-	-
12α	1.45	1.49	1.49	1.28	1.30	1.61	1.72
12β	2.09	2.36	[f]	2.20	2.21	2.27	2.16
14	1.17	1.54	[f]	1.59	1.61	1.48	1.84
15α	1.72	1.91	1.89	2.01	2.00	2.18	2.14
15β	1.33	1.45	[f]	1.37	1.41	1.54	1.64
16α	1.72	1.61	[f]	ca 1.85-1.92	ca 1.9	2.10	2.02
16β	2.19	2.16	[f]	ca 1.85-1.92	ca 1.9	2.31	2.29
17	2.54	2.59	ca 2.6	3.30	ca 3.30	2.95	2.95
18	0.70	5.40	5.43	4.90	4.90	5.54	5.66
19	1.40	1.33	1.47	1.29	1.45	1.31	1.48
21	2.13	3.47 3.40[j]	3.46 3.39[j]	4.32 4.19[k]	4.33 4.20[k]	-	-

[a] δ, ppm. In CD_3OD unless otherwise indicated, relative to δ 3.30 for residual $CD_2\underline{H}OD$ as reference value.
[b] 6β-Hydroxyprogesterone.
[c] In $CDCl_3$, relative to Me_4Si as internal standard.
[d] δ values are based on those in reference 8, but are increased by 0.1 ppm to conform to the reference value given in footnote a.
[e] Major form (18,20-hemiacetal 6).
[f] Sample was insufficient to allow accurate determination.
[g] (t, $J_{6\alpha,7\alpha} \approx J_{6\alpha,7\beta} \approx 3$ Hz).
[h] (m, $J_{6\alpha,7\alpha} = 3$ Hz; $J_{7\alpha,7\beta} = 14$ Hz; $J_{7\alpha,8} = 11.5$ Hz).
[i] (dt, $J_{6\alpha,7\beta} = 3$ Hz; $J_{7\alpha,7\beta} = 14$ Hz; $J_{7\beta,8} = 3$ Hz).
[j] (dd, $J = 11$ Hz). [k] (dd, $J = 20$ Hz).

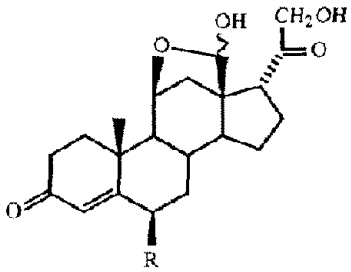


1 R = H



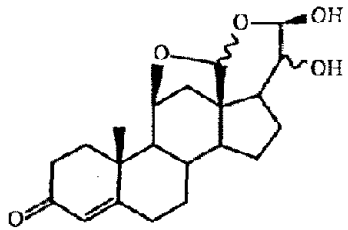
2 R = H

6 R = OH



3 R = H

8 R = OH



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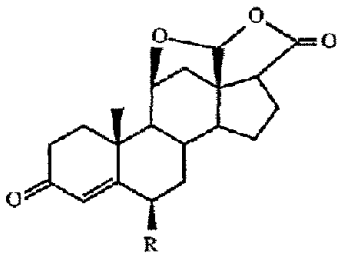

5 R = H

7 R = OH

we have extended the range of fully-assigned ^{1}H NMR spectra to include those of 17-isoaldosterone 3, 'apoaldosterone' 4 (7), and 'aldosterone γ-lactone' 5. We also had available a series of fully assigned spectra (to be published) for hydroxylated derivatives of progesterone, including 6β-hydroxyprogesterone. ^{1}H NMR data are collected in Table 1.

In the present investigation, both the 2.7-min and 5.0-min fractions showed features indicative of the 6β-hydroxy-4-en-3-one system, as found in the spectrum of 6β-hydroxy-progesterone. A characteristic poorly-resolved triplet at approximately δ 4.18 was assigned to the equatorial 6α-proton (9), which is spin-coupled only to the two protons of the 7-methylene group (COSY). A strong downfield shift of the 19-H_3 signal, to approximately δ 1.45, (10) and the appearance of the 4-H signal as a singlet at δ 5.68, in contrast to the narrow doublet which results from allylic 4-H/6β-H coupling in the absence of a 6β-substituent (11), provided further evidence for 6β-hydroxylation.

The spectra of the two products differed in the regions associated with protons around rings C and D, and particularly at the C-12, C-16, C-17, and C-21 positions. Like aldosterone itself (8), the first-eluted product (2.7 min) showed two double doublets, one at δ 4.31 and δ 4.12 (J 18 Hz), and the other at δ3.36 and 3.29 (J 11 Hz), characteristic of the 21-CH_2 protons in the 20-oxo form (1) and the 18,20-hemiacetal form (2), respectively. Relative signal intensities indicated that

the proportions of these forms present in solution in CD_3OD were in approximate ratio 1:3. Other recognisable features of the spectra (Table 1) support the conclusion that this fraction was essentially 6β-hydroxyaldosterone, present as an equilibrating mixture of the 20-oxo and 18,20-hemiacetal forms. Some relatively unimportant regions of the spectrum were obscured by stronger peaks associated with the solvent and traces of non-steroidal contaminants, so that a full assignment of the spectrum was not feasible.

To confirm the identification of 6β-hydroxyaldosterone 6, the whole sample was subjected to degradation with aqueous methanolic sodium metaperiodate (7), when it gave the 6β-hydroxy derivative 7 of 'aldosterone γ-lactone'. The identity of this product was confirmed by an independent chemical synthesis (see below) and comparison of the two samples, which showed identical TLC and HPLC mobilities. More significantly, their 500 MHz ^1H NMR spectra showed 'fingerprint' identity.

The second product (5.0 min) was recognized as having the 17-iso structure from spectral comparison with 17-isoaldosterone (7) itself. ^1H NMR spectra were obtained for solutions in CD_3OD and in C_5D_5N, to take advantage of a particularly large solvent shift of the signal associated with the 17β-proton in the 17-iso series (unpublished observation). In CD_3OD that signal is largely masked by the methanol peak centred at δ 3.3, although with larger amounts of material it

can usually be observed as a double doublet (four peaks of essentially equal intensity and spacing) centred at δ 3.2. That signal is shifted to δ 3.92 in C_5D_5N for 17-isoaldosterone itself, and is then seen clearly in a region unaffected by other signals. The spectrum of 6β-hydroxy-17-isoaldosterone in C_5D_5N showed this characteristic double-doublet centred at δ 3.86. The 17-iso structure is further indicated by the signals arising from the 21-CH_2 protons, which form a strong double doublet at δ 4.23 and δ 4.10 (J=20 Hz). 18,20-Hemiacetal formation is prevented in the 17-iso series by the geometric dispositions of C-18 and C-20 on opposite faces of the molecule; 17-iso compounds are therefore wholly in the 20-oxo form, with no signal in the region of δ 3.3 where the 21-CH_2 proton signals of the hemiacetal tautomer of aldosterone itself are observed. Table 1 includes a full assignment of the ^1H NMR signals of 17-isoaldosterone, achieved with the aid of a 2D homonuclear shift correlated (COSY) spectrum, and by reference to our published data (8) for the spectrum of aldosterone itself to assist with recognition of signals from protons remote from the side-chain. There were very close resemblances in all parts unaffected by 6-hydroxylation, between the spectra of 17-isoaldosterone and the liver metabolite now identified as its 6β-hydroxy derivative 8.

Both 17-isoaldosterone 3 and apoaldosterone 4 can arise from aldosterone by chemical isomerization in weakly alkaline media (e.g., 0.03 M $KHCO_3$) (7). Close examination of

the NMR spectra of the 6β-hydroxylated metabolite fractions failed to reveal any additional signals which might have indicated the presence of traces of 6β-hydroxy-apoaldosterone. To exclude the possibility that the isolated 6β-hydroxy-17-isoaldosterone was merely an artifact resulting from contact of aldosterone with the incubation medium, or with the solvents or chromatographic packings used during its isolation, we carried out a control experiment (see Materials and Methods). No significant isomerization of aldosterone occurred under these conditions, the aldosterone being recovered essentially unchanged. The isomerization and stability of the hydroxylated derivatives of aldosterone are currently being studied in our laboratories.

Synthesis '6β-hydroxyaldosterone γ-lactone'

Reported methods for the conversion of steroidal 4-en-3-ones into their 6β-hydroxy derivatives involve formation of the enol (3,5-diene) derivative, followed by an oxidative step using oxygen in the presence of strong light or a peroxy-acid in aqueous medium.

We chose to use another variation developed in our own laboratory, employing iodosobenzene as oxidant. Enol acetylation of aldosterone γ-lactone 5, followed by oxidation with iodosobenzene in aqueous dioxan and chromatographic separation, gave the 6β-hydroxy-lactone derivative 7 in 50% yield. Its structure, and particularly the configuration at C-6, were confirmed by ^{1}H NMR including a COSY spectrum

(Table 1), which showed the expected features closely matching those of aldosterone γ-lactone for rings C and D, and those of 6β-hydroxyprogesterone for rings A and B.

DISCUSSION

From our earlier in vitro studies several polar NMA, with a wide range of polarities, were shown to be synthesized by microsomal preparations from liver of male rats. The majority of these polar derivatives of aldosterone were shown to be products of cytochrome P-450-dependent enzymes (3). In these earlier studies the major metabolites migrated within regions we have designated A, B, and C (as above) and all have similar retention times on the high resolution HPLC systems when compared to those formed from physiological quantities of [^{3}H]aldosterone administered in vivo to male rats.

In the studies reported here, the major product which migrates in region B has been shown to contain 6β-hydroxy-aldosterone. Significant quantities of 6β-hydroxy-17-iso-aldosterone were also shown to be present, as well as some other hydroxylated components which have yet to be fully identified. Several other polar NMA were also isolated chromatographically in the A and C regions, as we had described before (3). 17-Isoaldosterone and apoaldosterone (7) standards migrate in the region A in our chromatographic system. The hydroxylated derivatives isolated from region A are currently being analyzed by NMR and will be reported later.

The occurrence of cytochrome P-450 6β-hydroxylation of

other steroids in liver of male rats has previously been reported and it is known that this enzyme can be induced by pre-treatment of the rats with phenobarbital and other drugs (12). Both 6β- and 6α-hydroxylated cortisol have been isolated from human urine, and high levels of 6β-hydroxycortisol have been measured in human urine, in pregnancy and toxemia, as well as in several other clinical conditions (13-16). Although the presence of 6β-hydroxycortisol was identified in human and other species in the early 1950's, no biological significance was associated with this steroid. Recent work by Watlington, and co-workers (17) has shown that 6β-hydroxycorticosterone is produced by the A6 cell line derived from toad kidney, and that this steroid can cause active Na^+ transport across confluent layers of the cells.

From the present studies it is now clear that aldosterone can also serve as a substrate for rat liver 6β-hydroxylase, or less probably that a different aldosterone-specific 6β-hydroxylase is present. The isomerization of aldosterone at C-17 to 17-isoaldosterone has been a problem common to many investigators working with aldosterone (18) and recently the concomitant isomerization to apoaldosterone has been reported (7,19). However, control experiments in the present studies afforded no evidence that the conditions or workup procedures used for the isolation of the products of incubation of aldosterone with the microsomal preparations lead to any significant conversion of aldosterone to 17-iso-

aldosterone. Further experiments are needed to determine whether 6β-hydroxylation proceeds or follows isomerization at C-17 in the formation of 6β-hydroxy-17-isoaldosterone; possibly both routes may be available.

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NOTE

For preceding paper in this series see Reference 8.

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