Biochemical Pharmacology, Vol. 19, pp. 1485–1492. Pergamon Press. 1970. Printed in Great Britain

16-OXYGENATION OF 17α-METHYLTESTOSTERONE IN RABBITS

TADASHI WATABE, SACHIKO YAGISHITA and SHOJI HARA

Division of Organic Chemistry, Tokyo College of Pharmacy,
Ueno-sakuragi, Taito-ku, Tokyo 110, Japan

(*Received* 9 *June* 1969; *accepted* 7 *October* 1969)

Abstract—Two polar metabolites with oxygen groups at 16-position were isolated from urine of male rabbits dosed with 17α-methyltestosterone and characterized as 17α-methyl-5β-androstane-3α, 16β, 17β-triol (Ia, metabolite A) and 3α, 17β-dihydroxy-17α-methyl-5β-androstan-16-one (IVa, metabolite B). Presence of the third polar metabolite was shown by thin-layer and gas chromatography, and it was identified as a 16-epimer of metabolite A, 17α-methyl-5β-androstane-3α,16α,17β-triol (Va, metabolite C). Metabolite A was excreted in the most significant amount, and metabolite C was almost in a negligible amount. No evidence was obtained that the animals excreted the corresponding 5α-isomers of these polar metabolites.

17α-METHYLTESTOSTERONE, which is well known as a more potent androgen than testosterone in oral therapy, has been demonstrated to be metabolized without splitting off its 17α-methyl group in rats[1] and by liver tissues of several kinds of animals, including rabbits.[2] Rongone and Segaloff have reported identification and excretion ratio of two tetrahydro metabolites of this steroid, isolated from urine of a woman with ovarian carcinoma upon oral administration of its large dose; the ratio of 5β and 5α diols, based on solids isolated, was about ten to one.[3] Their evidence for the ratio of the diols shows a marked contrast to that obtained with testosterone which is recognized to be excreted in urine as about one to one ratio of 5α and 5β isomers.[4] This encouraged us to make further investigation on the *in vivo* metabolism of 17α-methyltestosterone.

METHODS AND MATERIALS

Administration of 17α-methyltestosterone. Each of five male albino rabbits, weighing 2·8–3 kg, was dosed every 48 hr for 4 days with 1 g of the steroid (Roussel–Uclaf, Paris), suspended in 10 ml of a 10% gum arabic solution, by oral administration.

Collection and extraction of urine. Urine was collected until 8 days after the first administration and stored at 2° under a thin layer of toluene. The pooled urine was adjusted to pH 5·0 with acetic acid, incubated with a calf-liver β-glucuronidase preparation (61 Fishman units/ml urine) at 38° for 4 days, saturated with sodium chloride, and extracted twice with two volumes of ethyl acetate containing 5% of isoamyl alcohol. The combined organic layer was washed with 1 N NaOH followed by 0·2 N HCl to eliminate acidic and basic materials. Usual work-up of the organic solution containing neutral materials yielded 3·8 g of a coloured gum.

Chromatography. Thin-layer chromatography (TLC) was carried out in the usual manner using glass plates coated with silica gel (Wakogel B-0; Wako Pure Chemicals Co., Tokyo) and solvent mixtures as indicated in the text. The chromatograms were visualized by spraying with concentrated sulfuric acid followed by heating. For column chromatography silica gel was also used (Wakogel C-200). Gas chromatography (GLC) was carried out on a Shimadzu GC-1C gas chromatograph equipped with a hydrogen flame ionization detector and a pyrex glass column packed with 1·5% SE-30 on Chromosorb W (60–80 mesh), 180 cm in length and 4 mm in internal diameter. The temperature of the detector bath was 250°, and the flow rate of nitrogen as a carrier gas was 30 ml/min. Trimethylsilylation of the steroids was carried out by the method previously described,[5] and the trimethylsilyl ethers were dissolved in the volume of dry acetone so that 1 μl contained 0·5 μg of each steroid.

Absorption spectra. i.r. spectra were recorded on a Hitachi i.r. spectrometer by the KBr pellet method, and n.m.r. spectra on a Varian HA 100 n.m.r. spectrometer using about 2% solution of each sample in $CDCl_3$ containing tetramethylsilane as an internal reference.

Preparation of derivatives (IIa, IIb, IIIa and IIIb) *of metabolite* A (Fig. 1). 16β,17β-Isopropylidenedioxy-17α-methyl-5β-androstan-3α-ol (IIa) and its benzoate (IIb): a suspension of urinary metabolite A (100 mg) in acetone (10 ml) containing 60% perchloric acid (1 drop) was stirred at room temperature until it made a homogenous solution, and the acid was neutralized with solid potassium carbonate. Usual work-up of the mixture gave a colourless gum (IIa) quantitatively which was found to be pure by TLC and GLC. IIb was obtained by the treatment of IIa with benzoyl chloride in pyridine as prisms (190 mg), recrystallized from acetone and methanol, m.p. 213–214° (Found: C, 77·02; H, 8·88. $C_{30}H_{42}O_4$ requires C, 77·21; H, 9·07); i.r. cm^{-1}: 1712 (ester C=O), 1266 (ester C–O), and 709 (aromatic CH).

16,17-Seco-17-methyl-5β-androstane-3,17-dion-16-oic acid (IIIa) and its methyl ester (IIIb): to a solution of metabolite A (100 mg) in acetic acid (5 ml) was added dropwise a solution of chromium trioxide (60 mg) in 80% acetic acid (10 ml) at 2° during 30 min under stirring. After 20 min, the mixture was treated with isopropyl alcohol and concentrated below 40° to yield a syrup. Usual work-up for extracting the acid material formed gave prisms (42 mg), recrystallized from acetone and methanol, m.p. 266–267° (Found: C, 71·86; H, 9·03. $C_{20}H_{30}O_4$ requires C, 71·81; H, 9·04); i.r. cm^{-1}: 3300 (OH), 1736 and 1698 (C=O) and 1168 (lactol C–O). The i.r. spectrum was suggestive that the acid exists as a lactol in a solid state. The methyl ester of the acid was obtained by the treatment with diazomethane in ether in the usual manner; n.m.r. ppm: singlets at 1·017 (10-CH_3), 1·048 (13-CH_3), 2·226 (13-$COCH_3$), and 3·657 ($COOCH_3$).

Sodium borohydride reduction of metabolite B (*Fig.* 1). To a solution of metabolite B (50 mg) in a mixture of methanol (5 ml) and 2 N NaOH (0·3 ml) was added 1·5 molar equivalent sodium borohydride, and the reaction mixture was stirred for 1 hr. The product obtained consisted of two triols, which were resolved by column chromatography, affording 24 mg and 18 mg of solids. The former was recrystallized from acetone and methanol to give prisms, m.p. 266–268°, turning into needles at 257–258°. The i.r. spectrum was identical with that of metabolite A, and no melting point depression was observed with their admixture. The latter solid was recrystallized from methanol to give prisms, 17α-methyl-5β-androstane-3α,16α,17β-triol (Va), m.p. 221–

222° (Found: C, 74·37; H, 10·54. $C_{20}H_{34}O_3$ requires C, 74·49; H, 10·63); i.r. cm^{-1}: 3416 (OH) and 1058 and 1039 (C–O); n.m.r. as a diacetate (Vb) ppm: 0·945 (3H, singlet, 13-CH_3), 0·975 (3H, singlet, 10-CH_3), 1·095 (3H, singlet, 17α-CH_3), 2·045 and 2·125 (3H, respectively, two singlets, 3α- and 16α-$OCOCH_3$), 4·743 (1H, septet, 3β-H), and 5·025 (1H, doublet, J = 9Hz, 16β-H).

Preparation of 17α-*methyl-5β-androstane-*3α,16β,17β-*triol* (Ia: *metabolite* A, *Fig.* 2). Introduction of an acetoxyl group specifically at 16β-position of 3α-hydroxy-5β-androstan-17-one (VI) was carried out by the method of Leeds *et al.*,[6] and the procedures used here were substantially the same as for their 5α-steroid series.

3α,17β-Diacetoxy-5β-androst-16-ene (VII) was prepared from VI[7] (5 g) and recrystallized from hexane to give prisms (3·4 g), m.p. 88–89° (Found: C, 73·93; H, 9·32. $C_{23}H_{34}O_4$ requires C, 73·76; H, 9·15); i.r. cm^{-1}: 1767 and 1734 (C=O), 1618 (C=C), 1255 and 1212 (ester C–O), and 835 and 825 (=CH).

3α,16β-Diacetoxy-5β-androstan-17-one (VIII) was prepared from VII (2 g) and obtained as a colourless gum (1·95 g); i.r. cm^{-1}: 1740 (C=O) and 1243 (ester C–O); n.m.r. ppm: 0·965 (6H, singlet, 10- and 13-CH_3), 2·030 and 2·120 (3H, respectively, two singlets, 3α- and 16β-$OCOCH_3$), and 4·926 (2H, multiplet, 3β- and 16α-H).

17α-Methyl-5β-androstane-3α,16β,17β-triol (Ia: metabolite A) was prepared from VIII (1 g) by the reaction with 4 molar equivalent methylmagnesium iodide in absolute ether and recrystallized from methanol and acetone to give prisms (0·58 g), m.p. 266–268°, turning into needles at 254–257°. The i.r. spectrum was identical with that of urinary metabolite A, and no melting point depression was observed with their admixture.

RESULTS

Isolation of polar metabolites. Presence of at least seven metabolites in the neutral urinary extract (2 g), obtained on administration of 17α-methyltestosterone (5·26 g) to male rabbits, was shown by TLC in benzene and acetone (4:1) at R_fs 0·12, 0·18, 0·27, 0·31, 0·37, 0·45 and 0·56 after cancelling spots due to naturally occurring materials in urine of untreated rabbits. Two authentic diols, 17α-methyl-5α- and -5β-androstane-3α,17β-diols, which were previously reported as urinary metabolites of this steroids,[3] were located at an area between R_fs 0·43 and 0·48 on the same plate.

The chromatogram suggested that among the metabolites more polar than the diols, the R_f 0·27 and 0·37 materials were major metabolites comparable to the diols located at R_f 0·45, and they were separated by column chromatography using benzene containing increasing amounts of acetone. Yields of the R_f 0·12, 0·27 and 0·37 materials were, on evaporation of the solvent, 21 mg and 307 mg as crystalline solids and 185 mg as a colourless gum, respectively.

The R_f 0·18 and 0·31 materials were obtained in minor yields and found to be complicated mixtures from their colouration on the chromatogram. They were, therefore, discarded.

Characterization and identification of polar metabolites. 1. *Metabolite* A (R_f 0·27 *material*). The crystalline solid isolated was pure by TLC and recrystallized from acetone and methanol to give prisms (260 mg), m.p. 266–268°, turning into needles at 254–257°. The analytical data indicated the metabolite to be a methylandrostane with three hydroxyl groups (Found: C, 74·65; H, 10·70. $C_{20}H_{34}O_3$ requires C, 74·49; H, 10·63), suggesting that it arose by the reduction of the carbonyl and olefinic groups of

the mother steroid and introduction of a hydroxyl group. The i.r. spectrum showed presence of only hydroxyl groups absorbing at 3436, 3344, 1060, 1038 and 1024 cm^{-1} as a characteristic functional group. That the 17α-methyl and 17β-hydroxyl groups remained intact was confirmed by acetylation of the metabolite with acetic anhydride in pyridine to a diacetate (Ib), m.p. 82–83°; i.r. cm^{-1}: 3497 (OH), 1742 and 1715 (C=O), and 1266 (ester C–O); n.m.r. ppm: singlet signals at 0·851 (13-CH_3), 0·944 (10-CH_3), 1·238 (17α-CH_3), 2·002 and 2·076 (two $OCOCH_3$) and a multiplet at 4·620 (2H, two CHOAc).

Location and configuration of the hydroxyl group introduced to the steroid nucleus *in vivo* was assigned to be 16β from the following facts: first, the metabolite gave an acetonide (IIa) readily and quantitatively by the treatment with acetone containing a catalytic amount of perchloric acid, and IIa was then converted to the corresponding monobenzoate (IIb) which showed no longer any absorption band due to a hydroxyl group. Secondly, oxidation of the metabolite with chromium trioxide in acetic acid gave a diketocarboxylic acid whose structure was depicted as IIIa from the results of its i.r. spectrum and elemental analysis and the n.m.r. spectrum of its methyl ester (IIIb). Detailed physical data of these materials are shown in the Materials.

Configuration of the 3-hydroxyl group and A/B ring junction were assigned as β and *cis*, respectively, by comparing the chemical shift value, 0·944 ppm, of the 10-methyl protons of metabolite A diacetate (Ib) with the calculated values of those of four possible 3-acetoxy-androstanes:[8] 0·817, 0·842, 0·950 and 0·983 ppm for the 3α–5α, 3β–5α, 3α–5β and 3β–5β systems, respectively. The assignment was based on the knowledge that ring D substituents give little influence on the chemical shift of the 10-methyl protons.[8] Thus, the structure of the metabolite was tentatively established as 17α-methyl-5β-androstane-3α,16β,17β-triol (Ia).

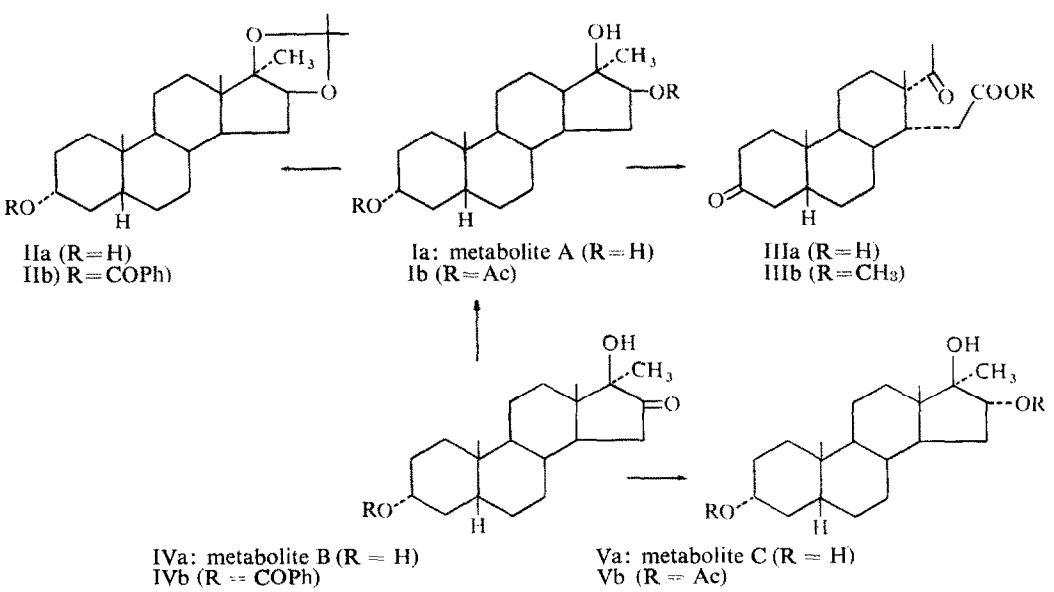


FIG. 1. Characterization of 17α-methyltestosterone metabolites.

The structural assignment was finally made by the synthesis of Ia from 3α-hydroxy-5β-androstan-17-one[7] (VI) as a starting material according to the method of Leeds *et al.*[6] for introducing an acetoxyl group specifically at 16β-position of a 17-ketosteroid. The synthetic pathway is illustrated in Fig. 2. Both urinary and synthetic specimens showed the same melting point, and no melting point depression was observed with their admixture. And their i.r. spectra were also superimposable on each other.

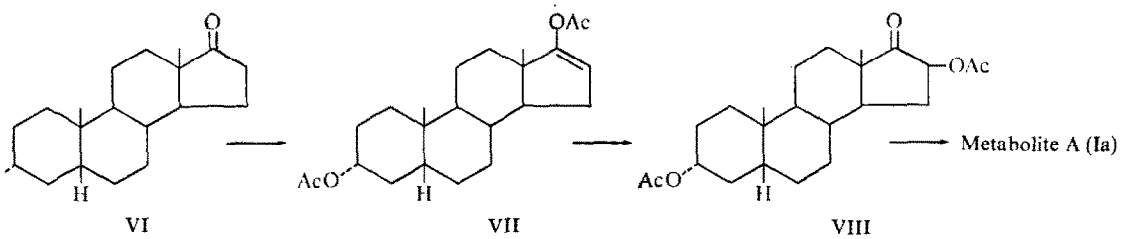


FIG. 2. Synthesis of metabolite A.

2. *Metabolite B (R_f 0·37 material).* Attempts to crystallize this material resulted in failure. However, it was pure enough to show a single peak at 11·3 min in the gas chromatogram obtained on 1·5% SE–30 at 210°. Treatment of the metabolite with benzoyl chloride in pyridine gave a crystalline monobenzoate, m.p. 182–183°, recrystallized from acetone and methanol (Found: C, 75·97; H, 8·64. $C_{27}H_{36}O_4$ requires C, 76·38; H, 8·55); i.r. cm^{-1}: 3489 (OH), 1754 (five-membered ring C=O), 1706 (ester C=O), and 1074 and 1023 (C–O); n.m.r. ppm: singlet signals at 0·803 (13-CH_3), 1·110 (10-CH_3), 1·200 (17α-CH_3), and 7·755 (C_6H_5), and a septet at 4·815 (CHOCOPh). These data, in addition to the fact that the metabolite did not form any acetonide under the same condition as was applied to metabolite A, suggested that it would be a 17α-methyl-androstane-3,17β-diol possessing one carbonyl group on its ring D.

Reduction of the metabolite with sodium borohydride gave a mixture of two triols (Fig. 1) which showed two spots at R_fs 0·27 and 0·12 on TLC obtained in benzene and acetone (4:1). After resolution by column chromatography, both of them were obtained as pure crystals, of which the higher R_f one was identified as Ia (metabolite A) by their superimposable i.r. spectra and the mixed melting point test. The other triol was reasonably characterized as a 16-epimer (Va) of metabolite A by elemental analysis, similarity of the i.r. spectrum to that of the latter, and recovery of the unchanged material on the treatment with acetone in the presence of an acid catalyst. Thus, the structure of the metabolite was elucidated as 3α,17β-dihydroxy-17α-methyl-5β-androstan-16-one (IVa).

3. *Metabolite C (R_f 0·12 material).* This material showed single spots on TLCs obtained in various solvent systems, whose R_f values were same as those of the 16-epimer (Va) of metabolite A, e.g. R_fs 0·23 in benzene and ethyl acetate (1:2) and 0·30 in chloroform containing 5% of methanol, but gave mixed crystals showing a broad melting point of 195–220° even after repeated recrystallizations from methanol or other solvents, suggesting obviously to be a mixture consisting of materials with similar

polarities. GLC of trimethylsilyl ethers of the mixture on a 1·5% SE–30 column indicated presence of four materials appearing at R_ts 9·1, 10·3, 12·6 and 13·2 min, of which the former two peaks were negligible, and the latter two almost same in their peak intensities. Among these well-isolated sharp peaks, the R_t 12·6 one was identified as the trimethylsilyl ether of the triol Va from the results of cochromatography at various column temperatures, and this was named metabolite C. Quantitative GLC data indicated that the mixture contained about 9 mg of metabolite C.

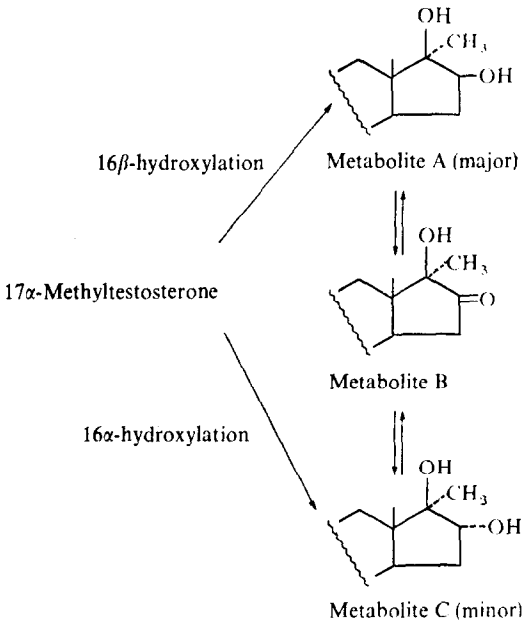
No further attempt was made to characterize the other major peak material, but it was shown by the i.r. spectrum of the mixture and acetonide formation test that it seemed to be a steroidal alcohol without any carbonyl and olefinic groups and *cis* glycol moiety in its molecule.

DISCUSSION

The present study demonstrates that about 90 per cent of metabolites more polar than methylandrostane-diols consists of metabolites A and B which have a 16β-hydroxyl and a 16-carbonyl groups, respectively, and the metabolite C, a 16-epimer of metabolite A, is produced in a very low yield. Percentage ratios of the polar metabolites to the urinary neutral extract are 15·4, 9·3 and 0·6 for metabolites A, B and C, respectively.

Though isolation of a 16β-hydroxy steroid has once been reported as a minor urinary metabolite following administration of testosterone to man, the present study would present the first evidence for the urinary excretion of the 16β-hydroxy steroid as one of the major metabolites. In comparison with the early results concerning the *in vivo* metabolism of estrogens as a typical and well-known instance for giving urinary 16-oxygenated steroids,[10] it should be noticed that 17α-methyltestosterone was converted to the 16β-hydroxy steroid in preference to the corresponding 16α-hydroxy one, for major components of urinary 16-hydroxy estrogens have a 16α-hydroxy group. Recently, various C_{19} steroids, including testosterone, have also been demonstrated to be directly hydroxylated at 16α-position by liver tissue preparations,[11–13] and their 16α-hydroxy derivatives have been found in significant amounts in umbilical cord blood at term[14–17] and placental tissue,[18] coming to the conclusion that they are important precursors for estriol.[19, 20] However, little information is available concerning 16β-hydroxylation of androgens and related C_{19} steroids *in vivo* as well as *in vitro*. For rationalization of the present results, the following alternative considerations may be possible: the 17α-methyl group hindered sterically the α-side attack of the 16-hydroxylase, leading to the preferential production of the 16β-hydroxylated steroid, otherwise most of the 16α-hydroxy steroid preferentially produced by the common α-side attack of the enzyme was converted to the 16β-hydroxy steroid through the 16-ketosteroid by oxidation followed by reduction steps. Limited to the estrogen series, a variety of informations regarding *in vivo* interconversion of 16α- and 16β-hydroxyl groups, which involves the 16-carbonyl group, are available, but little is known about the conversion of 16α-hydroxy C_{19} steroids to 16β-hydroxy ones. In this connection, Fishman *et al.* have recently demonstrated that the equilibrium between estriol and 16-epiestriol inclines remarkably toward the former *in vivo*.[21] Assuming that a similar equilibrium exists between metabolites A and C *via* B in the animal bodies and is mediated by the same or similar enzyme system as for the estrogens, the preferential production of metabolite A would be attributable to a steric role of the

17α-methyl group on reduction of the 16-ketosteroid or dehydrogenation of the 16-hydroxy steroids.



The polar metabolites of 17α-methyltestosterone isolated have all 5β-configuration, and none of the corresponding 5α-isomers was found in the urinary extract. Preferential formation of a 5β-diol has already been demonstrated by Rongone and Segaloff upon oral administration of a large dose of this steroid to a woman with ovarian carcinoma.[3] On the contrary, testosterone is known to give approximately equal ratio of 5α and 5β diol isomers *in vivo*.[4] It is of interest that both investigations on 17α-methyltestosterone came to similar results regarding the reduction of the double bond. This may be strongly suggestive that the 17α-methyl group also influences the reduction of the double bond *in vivo*.

Acknowledgements—The authors wish to thank members of Analytical Centers of Tokyo College of Pharmacy and of Research Institute of Kowa Co., Tokyo, for elemental analyses.

REFERENCES

1. P. M. Hyde, Wm. H. Eliott, E. A. Doisy, Jr. and E. A. Doisy, *J. biol. Chem.* **208**, 521 (1954).
2. B. H. Levedahl and L. T. Samuels, *J. biol. Chem.* **186**, 857 (1950).
3. E. L. Rongone and A. Segaloff, *J. biol. Chem.* **237**, 1066 (1962)
4. R. I. Dorfman, *Recent Progr. Horm. Res.* **9**, 5 (1954).
5. T. Luukkainen, W. J. A. VandenHeuvel, E. O. A. Haachi and E. C. Horning, *Biochim. biophys. Acta* **52**, 599 (1961).
6. N. S. Leeds, D. K. Fukushima and T. F. Gallagher, *J. Am. Chem. Soc.* **76**, 2943 (1954).
7. E. Elisberg, H. Vanderhaeghe and T. F. Gallagher, *J. Am. Chem. Soc.* **74**, 2814 (1952).
8. N. S. Bhacca and D. H. Williams, in *Application of NMR Spectroscopy in Organic Chemistry* p. 19. Holden-Day, Amsterdam (1964).

9. K. FORTHERBY, *Biochem. J.* **67**, 25 (1957).
10. R. I. DORFMAN and F. UNGAR, in *Metabolism of Steroid Hormones*, p. 292. Academic Press, New York (1965).
11. A. H. CÓNNEY and A. KLUTCH, *J. biol. Chem.* **238**, 1611 (1963).
12. A. CÓLAS, *Biochem. J.* **82**, 390 (1962).
13. W. L. HEINRICHS, H. H. FEDER and A. CÓLAS, *Steroids* **7**, 91 (1966).
14. W. E. EASTERLING, JR., H. H. SIMMER, W. J. DIGNAM, M. V. FLANKLAND and F. NAFTOLIN, *Steroids* **8**, 157 (1966).
15. A. CÓLAS, W. L. HEINRICHS and H. J. TATUM, *Steroids* **3**, 417 (1964).
16. H. H. SIMMER, M. W. FLANKLAND, M. GREISPEL and G. ZLONTONIK, *Steroids* **11**, 13 (1968).
17. H. G. MAGENDANTZ and K. J. RYAN, *J. Clin. Endocrinol. Metab.* **22**, 861 (1962).
18. R. NEHER and G. STARK, *Experientia* **17**, 510 (1961).
19. H. G. MAGENDANTZ and K. J. RYAN, *J. Clin. Endocrinol. Metab.* **24**, 1155 (1964).
20. S. DELL'ACQUA, S. MANCUSO, G. ERICKSSON, J. L. RUSE, S. SOLOMON and E. DICZFALUSY, *Acta Endocrinol.* **55**, 401 (1967).
21. J. FISHMAN, B. ZUMOFF, L. HELLMAN and T. F. GALLAGHER, *Steroids* **11**, 337 (1968).