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Pregnenolone-17α-^{3}H. Synthesis and study of label distribution

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SUMMARY

Pregnenolone-17α -^{3}H was synthesized by reaction of 17α-bromo-pregnenolone with tritiated acetic acid ($CH_3CO_2\ ^3H$) and zinc metal dust. The distribution of the label in the product was studied; about 98 % was at the 17-position and the remainder was randomly distributed on the steroids nucleus. It was also found that when pregnenolone was stirred with deuterated acetic acid ($CH_3CO_2\ ^2H$) and zinc metal dust for a longer period of time (22 hr) and in relatively high concentration, the protons of the C_{21}-methyl group (~57 %) were exchanged for deuterium.

For metabolic studies involving the biosynthesis of testosterone from pregnane precursors, it was necessary to synthesize 3β-hydroxy-5-pregnen-20-one-[17α-^{3}H] (pregnenolone-17α-^{3}H, **1**). This was accomplished by the stereospecific reaction [1] of 17α-bromopregnenolone [2] with zinc metal dust and tritiated acetic acid ($CH_3CO_2{}^3H$).

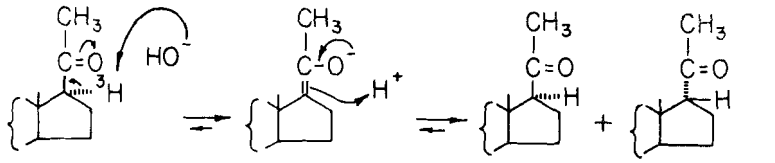
When **1** was used as substrate for incubations, some tritium was unexpectedly observed in certain isolated C_{19}-metabolites. To determine whether this tritium resulted from metabolic transformation or from labeling of the steroid nucleus during the preparation of **1**, the distribution of its tritium label was investigated.

Initially **1** was equilibrated * with potassium hydroxide in methanol-water to eliminate tritium from the 17- and 21-positions.

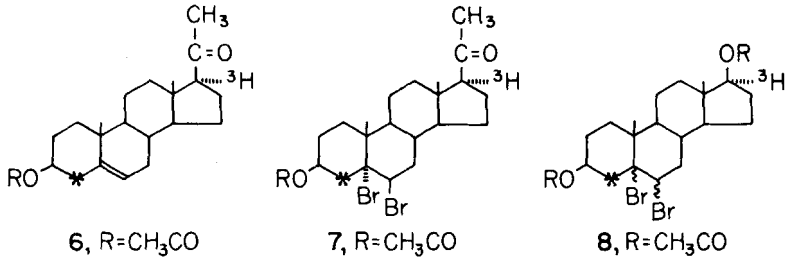
The product of equilibration was a mixture [3] of pregnenolone **3a** and isopregnenolone (**4a**), which were separated by fractional crystallization of

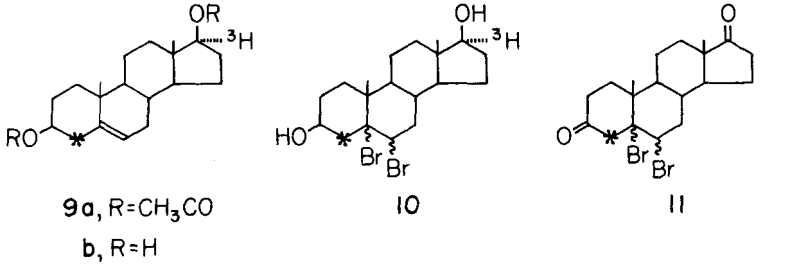
* Pregnenolone-4-^{14}C was used as internal standard.

their acetates (**3b** and **4b**). Compound **3b** retained about 2 % of the original tritium label indicating that some tritium was distributed in the steroidal nucleus. Oxidation of **3b** with *tert*-butyl chromate [4] to 7-oxopregnenolone acetate [5] **5** showed that some of the label (~0.6 %) was located at the C-7 position (Table I).



To show that the label was substantially at 17- rather than the 21-position, compound **1** *, after protection *via* acetylation and bromination (**7**), was subjected to the Baeyer-Villiger reaction [6]. Debromination of the Baeyer-Villiger product **8**, gave the diacetate **9a** which had retained the original tritium label. Deacetylation of **9a** resulted in the diol **9b** which also retained all the tritium label, confirming that the label was not at the C-21 position.





* Pregnenolone-4-^{14}C was used as internal standard.

TABLE I. Tritium distribution in pregnenolone-17α-^{3}H.[a] Equilibration and oxidation experiments.

Compound	Cryst. #	Specific activity[b] dpm/μmole		^{3}H/^{14}C-ratio	Tritium %
		^{3}H	^{14}C		
Pregnenolone-[4-^{14}C]-[17α-^{3}H][c] (**2**)	1	100,981	1,554		
	2	95,917	1,532		
	3	101,904	1,555		
	Avg.	99,600	1,547	64.4	100.0
Pregnenolone-^{3}H-[4-^{14}C] acetate[d] (**3b**)	1	1,350	1,094		
	2	1,292	1,107		
	3	1,259	1,157		
	Avg.	1,300	1,119	1.2	1.8
7-Oxopregnenolone-^{3}H-[4-^{14}C] acetate[d] (**5**)	1	819	994		
	2	773	1,028		
	3	758	959		
	Avg.	783	994	0.8	1.2

[a] Pregnenolone-4-^{14}C was used as the internal recovery standard.
[b] Data for three consecutive recrystallizations and their average values are reported throughout.
[c] Recrystallized from methanol-water.
[d] Recrystallized from acetone-pentane.

Further confirmation that the label was at the 17-position was achieved by oxidation of **9b**, after protecting the double bond with bromine; subsequent debromination gave 4-androstene-3,17-dione (**12**) which retained about 1 % of the original label (Table II).

Although tritium was not found at C-21, prolonged reaction under conditions used for preparation of **1** may introduce substantial quantities of label. This was shown by treatment of pregnenolone with zinc metal dust and deuterioacetic acid, when 57 % of C-21 hydrogen was exchanged for deuterium as indicated by nmr spectroscopy ** (Fig. 1).

This study indicates that although most of the tritium label is found at the 17-position in **1** (~98 %), there is some randomly distributed label in the nucleus and this must be taken into account when using the steroid for metabolic studies.

** J. Holtzman and H. M. Fales (*J. Am. Chem. Soc.*, **89** : 708 (1967) showed that total exchange at C-21 in 17α-hydroxyprogesterone occurs in deuterioacetic acid and toluene-p-sulfonic acid overnight at room temperature.

TABLE II. Tritium distribution in pregnenolone-17α-^{3}H.[a] The Baeyer-Villiger reaction.

Compound [b]	Cryst. #	Specific activity[c] dpm/μmole		^{3}H/^{14}C-ratio
		^{3}H	^{14}C	
Pregnenolone-[4-^{14}C]-[17α-^{3}HC] acetate (**6**)	1	6,161	585	
	2	6,415	606	
	3	6,175	597	
	Avg.	6,250	596	10.49
5α,6β-Dibromopregnenolone-[4-^{14}C]-[17α-^{3}H] acetate (**7**)	1	6,670	641	
	2	6,285	615	
	3	6,581	631	
	Avg.	6,512	629	10.35
5-Androstene-3β,17β-diol-[4-^{14}C]-[17α-^{3}H] diacetate (**9a**)	1	6,290	555	
	2	6,168	553	
	3	5,797	525	
	Avg.	6,085	544	11.19
5-Androstene-3β,17β-diol-[4-^{14}C]-[17α-^{3}H] (**9b**)	1	6,881	588	
	2	7,076	594	
	3	7,190	583	
	Avg.	7,049	588	11.99
4-Androstene-3,17-dione-^{3}H-[4-^{14}C][d] (**12**)	1	(48)[d]	485	
	2	(69)	497	
	3	(58)	474	
	Avg.	(58)	485	(0.12)

[a] Pregnenolone-4-^{14}C was used as the internal recovery standard.

[b] The compounds were recrystallized from acetone-pentane.

[c] Data for three consecutive recrystallizations and their average values are reported throughout.

[d] Too little tritium activity was available in each crystallization to attain sequentially acceptable mean specific activity data.

EXPERIMENTAL SECTION

Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Nuclear magnetic resonance (nmr) spectra were obtained with a Varian A-60A spectrometer using deuteriochloroform solutions; chemical shifts are reported in parts per million (ppm) on the δ scale (tetramethylsilane = 0). Infrared spectra were recorded with a Perkin-Elmer 257 spectro-

photometer using chloroform solutions. The ultraviolet spectrum was obtained with a Cary Model 11MS spectrophotometer. Optical rotation was determined using a Zeiss Winkel polarimeter. Elemental analysis was obtained from Micro-Tech Laboratories, Inc. Skokie, Ill. Silica gel GF254 was used for thin layer chromatography (t. l. c.); 250 μ layers were used for analytical purposes and 1 mm thick layers for preparative work.

Pregnenolone-17α-^{3}H (**1**). — To a stirred solution of 17α-bromopregnenolone [2] (400 mg) in dry ether (25 ml) was added zinc powder (2.5 g, Mallinckrodt Chem. Works, St. Louis, Mo.) and tritiated acetic acid (CH_3-CO_2^{3}H, 1.0 Ci/mmole; 120 mg, New England Nuclear Corp., Boston, Mass.) in dry ether (5 ml). After 2-3/4 hr glacial acetic acid (0.2 ml) was added, and the reaction was continued for a further 45 min. T. l. c. then indicated that the starting bromosteroid had been consumed. The suspension was filtered, and the remaining solid material was washed exhaustively with dry ether. The filtrate was evaporated to dryness, and chloroform (25 ml) was added. The solution was filtered, evaporated to dryness, and the residue was chromato-

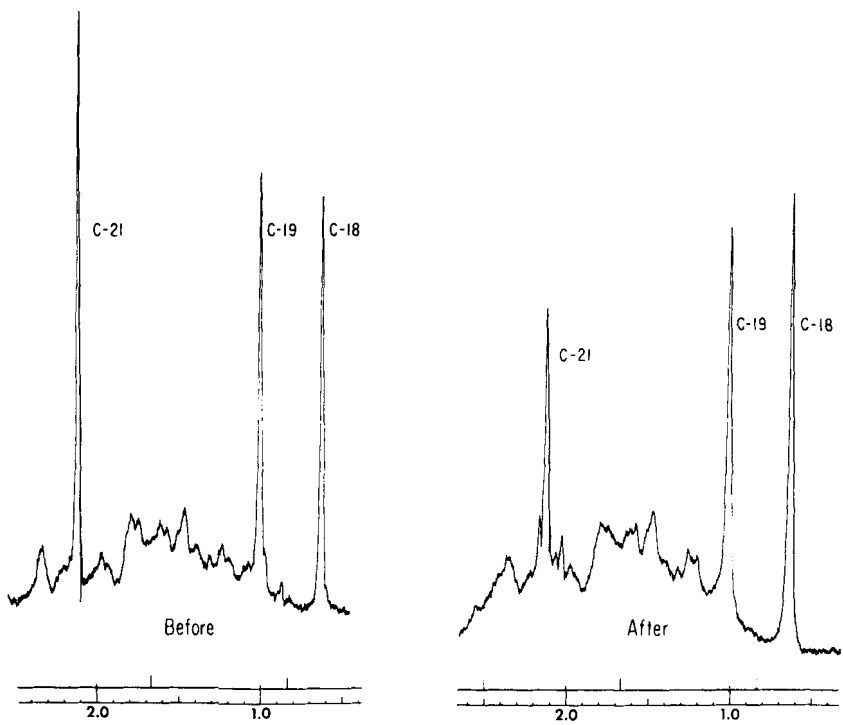


FIG. 1. Methyl resonances (C-18, C-19 and C-21) of pregnenolone before and after treatment with acetic acid-d and zinc metal dust for 22 hr.

graphed on a silica gel column using benzene as the eluent. The product was recrystallized twice from ethanol to give 277 mg of pregnenolone-17α-^{3}H, **1** (s.a. = 9.33 μCi/μmole), mp 189-191° (lit. [3] pregnenolone, mp 190°); $\nu_{max}^{CHCl_3}$ 3,595 (OH), 1,705 cm^{-1} (C = 0); nmr ($CDCl_3$) δ, 0.64 (18-H), 1.02 (19-H), 2.13 (21-H), 5.37 ppm (6-H). Radiochemical homogeneity was shown by paper chromatography (propyleneglycol-methylcyclohexane, 1 : 1, v/v) [7].

Distribution of the tritium label. (a) *Equilibration.* — A mixture of **1** (2.6 mg, 1.67×10^8 dpm), pregnenolone-4-^{14}C * (7.0 μg 2.57×10^6 dpm, New England Nuclear Corp., Boston, Mass.) and unlabeled pregnenolone (530 mg) was prepared. Five mg of this dual labeled pregnenolone **2** were used for recrystallization to constant specific activity (Table I). The remainder was dissolved in a warm solution of methanol (90 ml), water (10 ml) and potassium hydroxide (1.0 g), and the resulting solution was refluxed for 3 hrs under nitrogen. After cooling, the solution was neutralized with acetic acid, and the methanol was evaporated under a stream of nitrogen at 45°. The aqueous suspension was extracted with chloroform (5 × 30 ml). The chloroform extract was washed with water (2 × 20 ml), dried over anhydrous sodium sulfate, filtered and evaporated as above. The equilibration product was a mixture of labeled pregnenolone **3a** (major product) and isopregnenolone **4a** (minor product) [3], with tritium removed from the 17-position. The product was dissolved in dry pyridine (7 ml) and acetic anhydride (2 ml) and left overnight in the dark, after which the solvents were evaporated *in vacuo*. Benzene (50 ml) was thrice added to the residue and evaporated. The crystalline product appeared homogenous by t. l. c. on charring and scanning (benzene-ethyl ether, 8 : 3, v/v). The nmr spectrum had, however, in addition to the signals corresponding to pregnenolone acetate, **3b**, at δ 0.65 (18-H), 1.04 (19-H), 2.04 (CH_3-CO_2-), 2.13 (21-H); 4.62 (3α-H) and 5.42 ppm (6-H), a signal corresponding to C_{18}-H of isopregnenolone acetate **4b** (δ = 0.94 ppm) [5b] in the amount of ~14 %. Labeled **3b** was separated from **4b** by fractional crystallization from methanol and had mp 143-145° (lit. [3] pregnenolone acetate, 146-147°), $\nu_{max}^{CHCl_3}$ 1,732 (acetate), 1,705 (C_{20}, C = 0), 1,265 cm^{-1} (C-O-C); nmr ($CDCl_3$) δ, 0.64 (18-H), 1.03 (19-H), 2.03 (CH_3CO_2-), 2.13 (21-H), 5.38 ppm (6-H). Five mg of **3b** was used for recrystallization to constant specific activity (Table I).

(b) *Oxidation of labeled pregnenolone acetate* (**3b**) *to 7-oxopregnenolone acetate* (**5**). — *tert*-Butyl chromate [4, 5b] (30 ml) was added to a solution of labeled **3b** (392 mg) in carbon tetrachloride (3.5 ml). After 2 hr at 50° acetic anhydride (4 ml) was added. Glacial acetic acid (1 ml) was added after 4.5 hr, and another 5 ml were added after 5.5 hr. The heating (50°) was continued for a total of 22.5 hr. After cooling, the excess of chromate reagent was destroyed with a 10 % oxalic acid solution in water (100 ml). The mixture was extracted

* Pregnenolone-4-^{14}C (52.39 μCi/μmole) was purified by paper chromatography before use (methyl cyclohexane-propyleneglycol, 1 : 1, v/v) [7].

4× with carbon tetrachloride; the extract was washed with 5 % sodium hydroxide solution (1 × 10 ml) and with water (2 × 20 ml), and then was dried over anhydrous sodium sulfate, filtered and evaporated *in vacuo*. The product was chromatographed on a silica gel column using benzene as eluent; this was followed by benzene-ethyl ether (1 : 1, v/v) where the oxosteroid **5** was eluted. After two recrystallizations from ethyl acetate, labeled **5** (190 mg) was obtained having mp 150-152° (lit. [5a] 7-oxopregnenolone acetate, mp 153-153.5°); $\nu_{max}^{CHCl_3}$ 1,735 (acetate C = O), 1,696 (C_{20}, C = O), 1,677, 1,640 (C = C—C = O), 1,260 cm^{-1} (C—O—C); λ_{max}^{MeOH} 236 nm, (ε = 12,600) (lit. [5a] λ_{max}^{MeOH} 236 nm, ε = 13,200); nmr ($CDCl_3$) δ, 0.68 (18-H), 1.24 (19-H), 2.07 (CH_3-CO_2-), 2.14 (21-H), 5.76 ppm (6-H). Five mg of **5** was recrystallized to constant specific activity (Table I).

Baeyer-Villiger side chain cleavage. (a) *Pregnenolone-[4-^{14}C]-[17α-^{3}H] acetate* (**6**). — Pregnenolone-17α-^{3}H, **1** (0.38 mg, 2.5 × 10^7 dpm) and pregnenolone-4-^{14}C (6.8 μg, 2.5 × 10^6 dpm) in benzene-ethanol (9 : 1, v/v) were mixed, and the solvent was evaporated; dry pyridine (1.6 ml) and acetic anhydride (0.4 ml) were added and the solution was left overnight in the dark, at room temperature. The solvent was evaporated under a stream of nitrogen at 45°, after which a mixture of chloroform-methanol (1 : 1, v/v) was twice added and evaporated. The dual labeled steroid was diluted with 1.44 g of pregnenolone acetate, mp 149-150° C (Mann Research Labs.) using chloroform-methanol (1 : 1, v/v) as the solvent. On evaporation, crystals were obtained, and five mg of **6** was used for recrystallization to constant specific activity (Table II).

(b) *5α,6β-Dibromopregnanolone-[4-^{14}C]-[17α-^{3}H] acetate* (**7**). — To a cooled solution of **6** (990 mg) in glacial acetic acid (40 ml) was added 2.4 ml of a mixture prepared with glacial acetic acid (22 ml), bromine (4.0 g), potassium acetate (4.0 g) and water (2 ml). After 1 min, methanol (10 ml) and then water (150 ml) were added with stirring, and a precipitate formed. Stirring was continued for 10 mins and then the precipitate was filtered out and washed thoroughly with water. The colorless precipitate was dissolved in methylene chloride (140 ml), washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness *in vacuo* at room temperature. Recrystallization from methylene chloride-methanol gave 1.05 g of **7**, mp 140-143°. The product showed the same characteristics as authentic 5α,6β-dibromopregnanolone acetate, mp 141-143° (heating rate 1°/30 sec, from methylene chloride-methanol); $[\alpha]_D^{25}$-15.3° (c. 1.0, $CHCl_3$); $\nu_{max}^{CHCl_3}$ 1,733 (acetate), 1,705 (C_{20}, C = O), 1,260 cm^{-1} (C—O—C); nmr ($CDCl_3$) δ, 0.67 (18-H), 1.45 (19-H), 2.05 (CH_3-CO_2-), 2.13 (21-H), 4.85 (6α-H), 5.52 ppm (3α-H).

Anal. : Calcd. for $C_{23}H_{34}Br_2O_3$: C, 53.14; H, 6.62; Br. 30.93.
Found : C, 53.30 : H, 6.61; Br. 30.83.

Five mg of **7** was used for recrystallization to constant specific activity (Table II).

(c) *5-Androstene-3β,17β-diol-[4-^{14}C]-[17α-^{3}H] diacetate* (**9a**). — *m*-Chloroperbenzoic acid (2.0 g) was added to a solution of **7** (1.0 g) in methylene chloride (200 ml), and the mixture was refluxed. After 80 hr the nmr spectrum of an aliquot indicated that the reaction was not complete. *m*-Chloroperbenzoic acid (1.0 g) was added, and refluxing was continued for a further 46 hrs, but the reaction was not complete as shown by nmr spectroscopy. The reaction mixture was washed 2× with a 10 % solution of sodium hydroxide and then 3× with water. After drying with anhydrous sodium sulfate, filtration and evaporation *in vacuo*, the resultant product was chromatographed on a column prepared with 80.0 g of silica gel and eluted with benzene. The first eluted fractions contained 587 mg of a mixture of mostly 5α,6β- and 5β,6α-dibromoandrostane-3β,17β-diol-[4-^{14}C]-[17α-^{3}H] diacetate, **8**, as shown by nmr spectroscopy [8]. The later fractions contained a more complex mixture. To the product from the first fractions dissolved in dry ethyl ether (100 ml), glacial acetic acid (1 ml) and zinc powder (1.3 g) were added, and the mixture was stirred for 1 hr. T. l. c. (chloroform-methanol 95 : 5, v/v) indicated the presence of one major compound. The reaction mixture was worked up by first filtering the ether solution through a short silica gel column and then washing thoroughly with chloroform. The solvent mixture was evaporated, and chloroform was added. After washing with water and drying with anhydrous sodium sulfate, the solution was filtered and evaporated *in vacuo*. The product showed, by nmr spectroscopy, to be a mixture of 5-androstene-3β,17β-diol-[4-^{14}C]-[17α-^{3}H] diacetate, **9a** (~80 %) and **6** (~20 %). These compounds were separated by column chromatography on silica gel, using benzene as the eluant. Recrystallization from methanol of the first eluted product afforded 175 mg of pure **9a**, mp 156-158° (lit. [9] 5-androstene-3β,17β-diol diacetate, mp 157-157.5°); $\nu_{max}^{CDCl_3}$ 1,730 (acetates C = O), 1,269 cm^{-1} (C—O—C); nmr ($CDCl_3$) δ, 0.81 (18-H), 1.04 (19-H), 2.04 (CH_3-CO_2-, 6 protons), 4.62 (3α-H), 5.40 ppm (6-H). Five mg of **9a** was used for recrystallization to constant specific activity (Table II).

(d) *5-Androstene-3β,17β-diol-[4-^{14}C]-[17α-^{3}H]* (**9b**). — To a solution of **9a** (168 mg) in dry ethyl ether (50 ml), was added a 5 % solution of sodium methoxide in methanol (5 ml) and the mixture was left for 3 hr at room temperature, in an atmosphere of nitrogen. The solution was neutralized with acetic acid, and water was added. The ethereal layer was washed 2× with water, dried with anhydrous sodium sulfate, filtered and evaporated to dryness. The product was purified by preparative t. l. c. (chloroform-ethyl ether 1 : 1, v/v). The plate was dried out and rerun using the same solvent system. The product, **9b**, was recrystallized from acetone-petroleum ether and had a mp 174-175° (lit. [9] 5-androstene-3β,17β-diol, mp 178-179°; $\nu_{max}^{CHCl_3}$ 3,595 cm^{-1} (OH); nmr ($CDCl_3$) δ, 0.77 (18-H), 1.03 (19-H), 5.37 ppm (6-H). Five mg of **9b** were used for recrystallization to constant specific activity (Table II).

(e) *4-Androstene-3,17-dione-^{3}H-[4-^{14}C]* (**12**). — To a solution of **9b** (100 mg) in glacial acetic acid (20 ml) was added 0.6 ml of a solution prepared

with bromine (1.0 g), glacial acetic acid (5.5 ml), potassium acetate (1.0 g) and water (0.5 ml). After 1 min acetone (3 ml) was added. After 2 min the solution was poured into ice-water (200 ml) where the dibromosteroid **10** separated. After filtration, the product was dissolved in methylene chloride, washed with water, dried over anhydrous sodium sulfate, filtered and evaporated to dryness to give 82 mg of a gummy product, 5ξ,6ξ-dibromoandrostane-3β,17β-diol-[4-^{14}C]-[17α-^{3}H], **10**. Chromic acid reagent [10] (0.2 ml) was added dropwise to a solution of this product in dry acetone (20 ml) while cooling; the orange color persisted for 3 min. Methanol (5 ml) was added and after 10 min, the solvent was partly evaporated *in vacuo* at room temperature. To the remainder, brine solution was added and then extracted 3× with ethyl acetate; this solution was dried over anhydrous sodium sulfate, filtered and evaporated to give 63 mg of 5ξ,6ξ-dibromoandrostane-3,17-dione-4-^{14}C, **11**. This product was dissolved in dry ether (30 ml); zinc powder (300 mg) and glacial acetic acid (0.2 ml) were added and stirred at room temperature. After 1.5 hrs the suspension was filtered through a short silica gel column. The solvent was evaporated and the product chromatographed on a preparative t. l. c. plate (chloroform-ethyl ether 1 : 1, v/v). The ultraviolet absorbing region, moving together with an authentic sample of 4-androstene-3,17-dione, was scraped out and then eluted extensively with ethyl ether and chloroform. The solvent was evaporated, and the residue was chromatographed on a short chromatographic column made of alumina grade III, using a mixture of benzene-petroleum ether (30-60°) (1 : 1, v/v) as eluent. The steroid was eluted while the colored impurities remained on the column. The product **12**, after recrystallization from acetone-pentane, had mp 168-170° (lit. [11] 4-androstene-3,17-dione, mp 167-169°, from acetone); $\nu_{max}^{CHCl_3}$ 1,743 (C_{17}, C = O), 1,685, 1,626 cm^{-1} (C = C—C = O). Five mg of **12** was recrystallized to constant specific activity (Table II).

Reaction of pregnenolone with acetic acid-d (CH_3-CO_2D) and zinc powder. — To a solution of pregnenolone (945 mg) in dry ethyl ether (350 ml), acetic acid-d (2 ml, 98 % enrichment, Mallinckrodt Chem. Works, St. Louis, Mo.) and zinc powder (5.0 g) were added with stirring. After 7 hr the suspension was filtered, and the solvent was evaporated. The nmr spectrum ($CDCl_3$) of the recovered pregnenolone was identical with that of an authentic sample. Part of this recovered pregnenolone (104 mg) was dissolved in acetic acid-d (5 ml); dry ethyl ether (7 ml) and zinc powder (1.6 g) were added and the suspension was stirred for 16 hrs, after which additional zinc powder (1.0 g) was added. Stirring was continued for a further 6 hr, and then pregnenolone was recovered as above. The nmr spectrum indicated that a decrease in the 21-H signal had occurred when compared with authentic pregnenolone. Using the 18-H resonance as the reference signal, it was found that approximately 57 % of 21-H had been exchanged with deuterium (Fig. 1).

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