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# BIOSYNTHESIS OF PREGNANE DERIVATIVES IN *STROPHANTHUS KOMBÉ*

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**Abstract**—The following radioactive pregnane derivatives were isolated from *Strophanthus kombé* plants after administration of progesterone-4-$^{14}$C: 5$\alpha$-pregnane-3,20-dione, 5$\alpha$-pregnan-3$\beta$-ol-20-one, 5$\beta$-pregnane-3,20-dione, 5$\beta$-pregnan-3$\beta$-ol-20-one, 5$\beta$-pregnane-3$\beta$,20$\beta$-diol, and 5$\beta$-pregnane-3$\beta$,5$\beta$-diol-20-one.

## INTRODUCTION

THE BIOGENESIS of *Digitalis* cardenolides is presently under investigation in several laboratories.[1,2] The following steps in the biosynthetic pathway have now been elucidated: acetate→mevalonate→cholesterol→pregnenolone→progesterone (I)→cardenolides, e.g. digitoxigenin (II).[3–8] The intermediates between progesterone and the cardenolides remain to be clarified, however. The only information on this subject is the finding that tritiated 5$\beta$-pregnane-3$\beta$,14$\beta$-diol-20-one was converted to digitoxigenin by *Digitalis lanata* plants.[6] We previously approached this problem by looking for possible intermediates among the metabolites of pregnenolone-4-$^{14}$C and progesterone-4-$^{14}$C in *D. lanata* plants.[7,8] One such compound, 5$\beta$-pregnane-3,20-dione (III), was found, but two of the major metabolites of progesterone were 5$\alpha$-pregnane-3,20-dione (IV) and 5$\alpha$-pregnan-3$\beta$-ol-20-one (V). The *Digitalis* cardenolides are 5$\beta$-compounds, and the only 5$\alpha$-steroids known to be present in this plant are sapogenins, which are $C_{27}$ compounds. The finding of the two 5$\alpha$-$C_{21}$ steroids was, therefore, surprising. We then decided to look in another species, *Strophanthus kombé*, for possible intermediates in cardenolide biosynthesis and for 5$\alpha$-pregnane derivatives. The *Strophanthus* cardenolides differ from those of *Digitalis* in being oxygenated at the 5 and, except for periplogenin, the 19 positions, but they are also biosynthesized from progesterone.[9]

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. Work conducted under a cooperative agreement with the California Institute of Technology. Requests for reprints should be addressed to Erich Heftmann.

[1] R. TSCHESCHE, in *Terpenoids in Plants* (edited by J. B. PRIDHAM) pp. 111–118, Academic Press, New York (1967).
[2] T. REICHSTEIN, *Naturwissenschaften* **54**, 53 (1967).
[3] G. M. JACOBSOHN and M. J. FREY, *J. Am. Chem. Soc.* **89**, 3338 (1967).
[4] E. CASPI and D. O. LEWIS, *Science* **156**, 519 (1967).
[5] E. CASPI and G. M. HORNBY, *Phytochem.* **7**, 423 (1968).
[6] R. TSCHESCHE, H. HULPKE and H. SCHOLTEN, *Z. Naturforsch.* **22b**, 677 (1967).
[7] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **6**, 1521 (1967).
[8] R. D. BENNETT, H. H. SAUER and E. HEFTMANN, *Phytochem.* **7**, 41 (1968).
[9] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.*, in press.

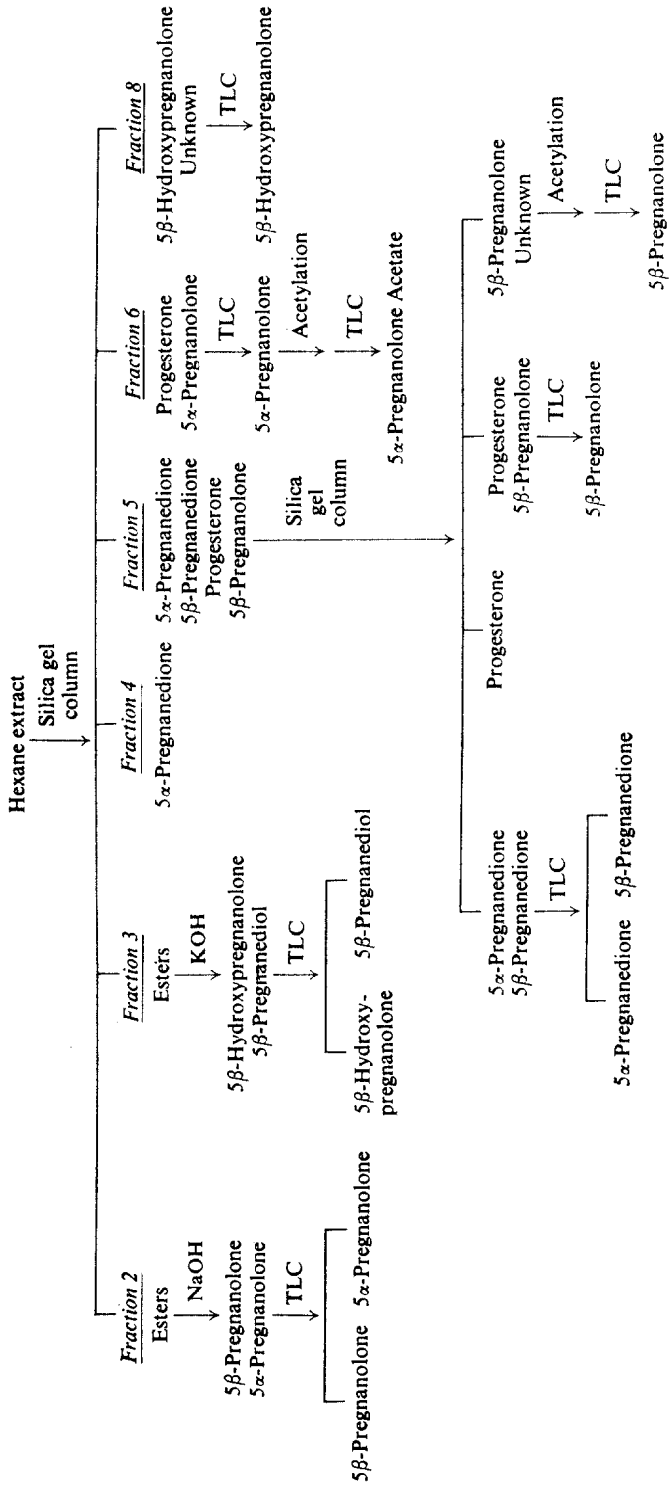
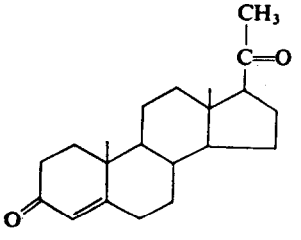
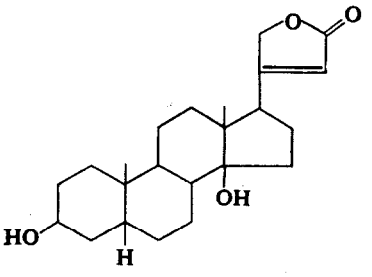


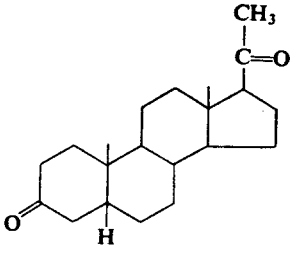

FIG. 1. SCHEME FOR ISOLATION OF RADIOACTIVE STEROIDS FROM HEXANE EXTRACT.



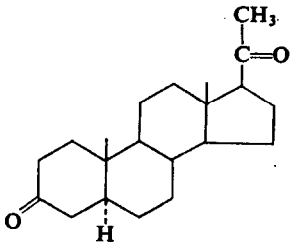
(I) Progesterone



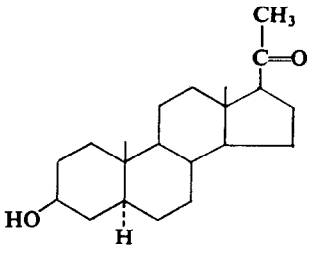
(II) Digitoxigenin



(III) 5β-Pregnanedione



(IV) 5α-Pregnanedione



(V) 5α-Pregnanolone

## RESULTS

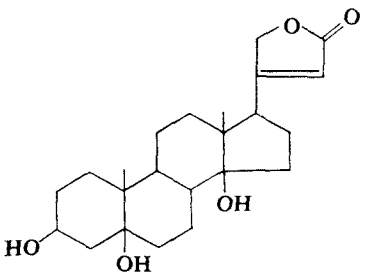
In the previous paper[9] we described the isolation of radioactive periplogenin (VI), strophanthidin (VII), and strophanthidol (VIII) from a dichloromethane extract of *Strophanthus kombé* plants that had been treated with progesterone-4-$^{14}$C. The hexane extract of these plants was then investigated by TLC. A scan of the chromatogram for radioactivity showed a major peak corresponding to progesterone and several minor peaks, both more and less polar than the latter. The hexane extract was then chromatographed on a silica gel column. Figure 1 outlines schematically the methods used to isolate the individual components of the column fractions.

The radioactive 5β-hydroxypregnanolone (IX) from Fraction 3 was diluted with carrier material (prepared by synthesis), crystallized to constant specific activity, acetylated, and further crystallized (Table 1, E). As additional proof of its identity, a portion of the radioactive material, with carrier, was oxidized to 5β-hydroxypregnanedione. This compound was then dehydrated to progesterone by heating with acetic acid.[10] In both cases, the radio-

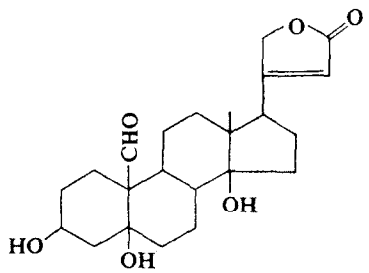
[10] A. LARDON, *Helv. Chim. Acta* **32**, 1517 (1949).

activity was shown by TLC to be associated with the known products. The dehydration to progesterone proves the presence of a hydroxyl group at the 5-position.

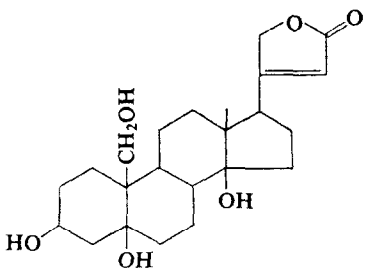
The 5α-pregnanedione (IV) from Fraction 4 and the 5β-pregnanedione (III) from Fraction 5 were each diluted with carrier material and crystallized to constant specific activity, after



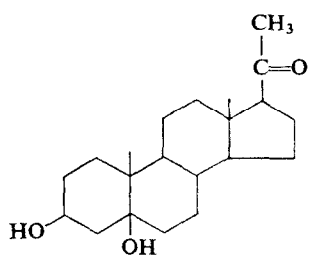

(VI) Periplogenin



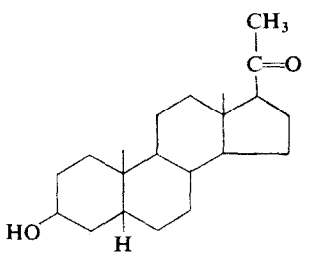

(VII) Strophanthidin



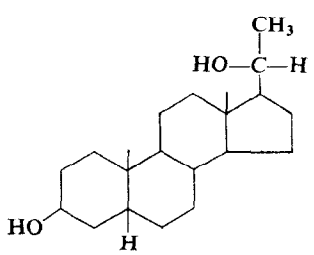

(VIII) Strophanthidol




(IX) 5β-Hydroxypregnanolone




(X) 5β-Pregnanolone




(XI) 5β-Pregnanediol

which they were reduced with sodium borohydride to 5α-pregnane-3β,20β-diol and 5β-pregnane-3α,20β-diol, respectively. These derivatives were isolated by preparative TLC and further crystallized (Table 1, A and B). The 5β-pregnanolone (X) acetate from Fraction 5

TABLE 1. RECRYSTALLIZATION OF STEROIDS TO CONSTANT SPECIFIC ACTIVITY*

| Compound | Solvent used for crystallization | Counts/min/μMole† |
|---|---|---|
| A. 5α-Pregnanedione | | 868±43 |
| | $CH_2Cl_2$–ether–light petrol. | 840±43 |
| | $CH_2Cl_2$–ether–light petrol. | 845±43 |
| | $CH_2Cl_2$–methanol | 857±44 |
| 5α-Pregnane-3β,20β-diol | Methanol | 838±42 |
| | Hexane–ether | 850±42 |
| B. 5β-Pregnanedione | | 194±7 |
| | Ether–light petrol. | 170±9 |
| | Ether–light petrol. | 161±6 |
| | Methanol–water | 160±6 |
| 5β-Pregnane-3α,20β-diol | Methanol | 173±9 |
| | Hexane–ether | 165±8 |
| C. 5α-Pregnanolone Acetate | | 2990±150 |
| | Ether–light petrol. | 3350±170 |
| | Ether–light petrol. | 3470±170 |
| | Methanol | 3550±180 |
| 5α-Pregnanolone | Ether–ethanol–light petrol. | 3350±170 |
| | Methanol | 3570±180 |
| D. 5β-Pregnanolone Acetate | | 248±12 |
| | Methanol | 255±13 |
| | Ether–light petrol. | 252±13 |
| 5β-Pregnanolone | Ether–light petrol. | 255±13 |
| | Methanol | 255±13 |
| E. 5β-Hydroxypregnanolone | | 1360±70 |
| | $CH_2Cl_2$–ether–light petrol. | 1130±60 |
| | $CH_2Cl_2$–ether–light petrol. | 1050±50 |
| | $CH_2Cl_2$–ether–light petrol. | 950±50 |
| | $CH_2Cl_2$–ether–light petrol. | 1020±50 |
| | $CH_2Cl_2$–light petrol. | 960±50 |
| | $CH_2Cl_2$–methanol | 960±50 |
| 5β-Hydroxypregnanolone Acetate | Ether–light petrol. | 870±40 |
| | Methanol–water | 910±40 |
| F. 5β-Pregnanediol | | 204±10 |
| | $CH_2Cl_2$–methanol–light petrol. | 176±9 |
| | $CH_2Cl_2$–methanol–light petrol. | 155±8 |
| | $CH_2Cl_2$–methanol–light petrol. | 156±8 |
| | Methanol | 147±8 |
| 5β-Pregnanediol Diacetate | Methanol | 148±7 |
| | Ethanol–water | 150±7 |

* Portions of 0·2 mg or less were plated from solution on ringed planchets over an area of 12·7 $cm^2$ and counted in duplicate on a Beckman Widebeta II instrument. Counter efficiency was 34 per cent and background was 2 counts/min.

† 90 per cent confidence level.

and the 5α-pregnanolone (V) acetate from Fraction 6 were each diluted with carrier material, crystallized to constant specific activity, hydrolyzed, and further crystallized (Table 1, C and D). The 5β-pregnanediol (XI) from Fraction 3 was diluted with carrier material, crystallized to constant specific activity, acetylated, and further crystallized (Table 1, F).

The total radioactivity of each of the isolated steroids, including the cardenolides,[9] is presented in Table 2. The radioactivities of the cardenolides were estimated from TLC scans, but the other values are based upon fractions which were radiochemically homogeneous by TLC. Where the presence of radioactive impurities was demonstrated by a decrease in specific activity during crystallization, the values were corrected accordingly. The tabulated values account for 5·68 per cent of the administered radioactivity.

TABLE 2. TOTAL RADIOACTIVITY OF INDIVIDUAL STEROIDS

| Compound | Counts/min × $10^{-3}$ | Per cent of original radioactivity |
|---|---|---|
| Periplogenin | 800* | 0·36 |
| Strophanthidin | 2000* | 0·90 |
| Strophanthidol | 1100* | 0·49 |
| 5α-Pregnanedione | 990 | 0·44 |
| 5β-Pregnanedione | 24 | 0·01 |
| 5α-Pregnanolone | 4400 | 1·97 |
| 5β-Pregnanolone | 1500 | 0·67 |
| 5β-Hydroxypregnanolone | 1170 | 0·52 |
| 5β-Pregnanediol | 720 | 0·32 |

* Approximate values.

## DISCUSSION

5β-Pregnanedione has now been isolated as a metabolite of progesterone-4-$^{14}$C from both *Digitalis lanata* and *Strophanthus kombé*, while 5β-pregnanolone and 5β-hydroxypregnanolone were found only in the latter plant. If 5β-pregnanedione immediately follows progesterone in the biosynthesis of cardenolides, as now seems likely,[5] the next step could be conversion to 5β-pregnanolone. Our failure to find radioactivity associated with the latter compound in *D. lanata* could be explained by a rapid turnover. 5β-Hydroxypregnanolone, on the other hand, would be expected as a cardenolide precursor only in *S. kombé*. Since we were unable to detect any radioactive 5β-hydroxypregnanedione in the latter plant, the following biosynthetic sequence seems reasonable: progesterone→5β-pregnanedione→5β-pregnanolone→5β-hydroxypregnanolone→cardenolides. This hypothesis is now being tested by readministering the radioactive pregnane derivatives to *S. kombé* plants. It seems unlikely that 5β-pregnanediol, which has not previously been isolated from plants, is involved in cardenolide biosynthesis.

As in the case of *D. lanata*, progesterone-4-$^{14}$C was converted by *S. kombé* to 5α-pregnanedione and 5α-pregnanolone. The latter was actually the major metabolite of progesterone. These results are especially surprising because 5α-steroids have not been detected in *Strophanthus kombé* heretofore, as far as we know. Graves and Smith[11] have previously found these two compounds as metabolites of progesterone in tissue cultures of eight other species of dicotyledons.

The radioactive 5α-pregnanolone, 5β-pregnanolone, and 5β-hydroxypregnanolone were found mainly in the form of esters. It was not possible to identify the acidic components of

[11] J. M. H. GRAVES and W. K. SMITH, *Nature* **214**, 1248 (1967).

the esters, but sterols are frequently found in plants as esters of fatty acids. The radioactivity of these compounds is higher than that of the two diones, which are incapable of forming esters. This suggests that the turnover of the esters may be slower and that they may be less active biosynthetically. The radioactivity of the 5α-pregnane derivatives was found to be higher than that of the corresponding 5β-steroids, a fact which also suggests that the former are not as actively metabolized as the latter.

## EXPERIMENTAL

### *Methods*

TLC techniques were as described previously,[12] except that a Packard Model 7201 scanner was also used. All chromatograms were run on Silica Gel G plates purchased from Analtech, Inc., Wilmington, Delaware.* For column chromatography, silica gel (particle size 0·05–0·2 mm) was obtained from Brinkmann Instruments, Westbury, New York. Aliquots of radioactive samples were counted on planchets at infinite thinness under a gas-flow detector (see Table 1, legend, for details). Melting points were taken on a Kofler block and are corrected.

### *Acetylation*

Acetylation reactions were carried out by a modification of the vapor phase technique of Norymberski and Riondel.[13] For microgram quantities, a small Weld pycnometer (Scientific Glass Apparatus Co., Inc., Bloomfield, New Jersey), with the capillary tube removed, was found convenient. The compound to be acetylated was deposited, by evaporation of a solution, as a thin film on the inside surface of the upper part. About 0·1 ml of pyridine and 0·1 ml of acetic anhydride were placed in the lower part, and the two parts were joined. The pycnometer was kept overnight at 25°, or placed in an oven for 1 hr at 90°. The upper part was then separated and freed of traces of reagents by placing it under a stream of nitrogen or under vacuum. For mg quantities, the sample was deposited in a thin film on the walls of a round-bottom flask, which was inverted and closed with a hollow stopper containing acetic anhydride and pyridine.

### *Synthesis of 5β-Hydroxypregnanolone*

$\Delta^4$-Pregnene-3β,20β-diol was prepared by reduction of progesterone with $NaBH_4$.[14] A solution of 40 mg of the crude product in 1·5 ml $CH_2Cl_2$ was treated with 40 mg *p*-nitroperbenzoic acid (K and K Laboratories, Hollywood, California) for 20 min at 25°. After addition of 3 ml of ether, the solution was extracted with 2 ml 10% $Na_2CO_3$. The extract was backwashed with 3 ml ether, and the two organic layers were washed with 1 ml water, combined, and evaporated, giving 41 mg of crude epoxide. TLC with ethyl acetate–cyclohexane (13 : 7) showed a major product slightly more polar than the starting material. By analogy with the epoxidation of $\Delta^4$-cholesten-3β-ol,[15] this should be the 4β,5β-epoxide.

The crude epoxide (32 mg) was acetylated, and the product was refluxed with 50 mg $LiAlH_4$ in 3 ml ether for 2 hr. The excess reagent was decomposed with ethyl acetate, followed by 1 ml water, the ether layer was separated, and the aqueous layer was extracted with two 3-ml portions of ether. The ether layers were washed with 1 ml water, combined, and evaporated, giving 32 mg of crude pregnane-3β,5β,20β-triol. Chromatographically homogeneous material was obtained by crystallization from acetone.

To a solution of 200 mg of pure triol in 30 ml acetone, cooled to 10°, was added 0·23 ml of Kiliani's solution (prepared by dissolving 2·6 g of chromic acid in 2·3 ml of conc. $H_2SO_4$ and 7 ml water). After 15 min at 10°, a few drops of methanol were added to reduce the remaining chromic acid. The solution was then diluted with 35 ml water, and the acetone was removed in vacuum. The aqueous residue was extracted with $CH_2Cl_2$ (3 × 30 ml). The extracts were washed with 5 ml water, 5 ml 10% $KHCO_3$, and two 5-ml portions of water, combined, and evaporated, giving 197 mg of crude oxidation product. TLC with $CH_2Cl_2$–methanol (47 : 3) showed three spots of approximately equal intensity. The most polar spot was due to the unreacted triol and the least polar one to 5β-hydroxypregnanedione, which was the only product when excess chromic acid was used for the oxidation. The middle spot corresponded to a reference sample of 5β-hydroxypregnanolone, obtained by degradation of periplogenin.[10] To destroy some pregnane-5β,20β-diol-3-one which cochromatographed with the 5α-hydroxypregnanolone, the crude product was refluxed with 15 ml acetic acid for 30 min.

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

[12] R. D. Bennett and E. Heftmann, *Phytochem.* **5**, 747 (1966).
[13] J. K. Norymberski and A. Riondel, *Experentia* **23**, 318 (1967).
[14] B. Camerino and C. G. Alberti, *Gazz. Chim. Ital.* **85**, 51 (1955).
[15] P. A. Plattner, H. Heusser and A. B. Kulkarni, *Helv. Chim. Acta* **32**, 265 (1949).

This treatment dehydrates 5β-hydroxy-3-ketones to the less polar $\Delta^4$-3-ketones,[10] but does not affect 3,5-diols. After evaporation of the acetic acid in vacuum, the 5β-hydroxypregnanolone was isolated by column chromatography on silica gel, followed by preparative TLC with $CH_2Cl_2$–methanol (47 : 3), giving 31 mg. An NMR spectrum, taken on a Varian A-60 instrument in $CDCl_3$, showed peaks at 2·15 ppm (—$COCH_3$), 1·01 ppm and 0·64 ppm (18- and 19-methyl). The acetate, after recrystallization from ether–light petroleum, melted at 144–147°. An authentic sample of 5β-hydroxypregnanolone acetate[10] melted at 143–147°, and a mixture of the two melted at 144–148°.

*Isolation of Steroids from Hexane Extract*

The hexane extract, obtained as described in the previous paper,[9] was chromatographed on a 50-g column of silica gel, packed into a chromatographic tube of 35 mm dia. as a slurry in cyclohexane. Fractions of 200 ml each were collected with the following eluents: 1, cyclohexane; 2–3, 10 per cent; 4–5, 20 per cent; 6–7, 30 per cent; 8–9, 50 per cent ethyl acetate in cyclohexane; and 10–11, ethyl acetate. The fractions were examined by TLC with cyclohexane–ethyl acetate (1 : 1).

Fraction 1 was devoid of radioactivity. Fraction 2 (313 mg, $7{\cdot}96 \times 10^6$ counts/min) was composed of nonpolar material which ran near the solvent front. One-fifth of Fraction 2 was refluxed with 20 ml 0·1 N NaOH in 80% methanol for 45 min. The mixture was diluted with 20 ml water, the methanol was evaporated in vacuum, and the aqueous residue was extracted with $CH_2Cl_2$ (3 × 20 ml.). The extracts were washed with two 5-ml portions of water, combined, and evaporated (49 mg, $1{\cdot}50 \times 10^6$ counts/min). TLC with $CH_2Cl_2$–methanol (93 : 7) showed a large peak near the front, apparently unhydrolyzed ester, and two smaller peaks, which corresponded chromatographically to 5α-pregnanolone and 5β-pregnanolone. Separation by preparative TLC with $CH_2Cl_2$–methanol (97 : 3) gave pure 5α-pregnanolone ($2{\cdot}80 \times 10^5$ counts/min) and 5β-pregnanolone ($2{\cdot}84 \times 10^5$ counts/min).

Fraction 3 (74 mg, $4{\cdot}56 \times 10^6$ counts/min) consisted of material which also ran near the solvent front but was slightly more polar than Fraction 2. One-tenth of Fraction 3 was treated with 1 ml of 5% KOH in 90% methanol for 3 days at 25°. The mixture was diluted with 2 ml water and extracted with $CH_2Cl_2$ (4 × 2 ml). The extracts were washed with four 1-ml portions of water, combined, and evaporated (3·7 mg, $4{\cdot}00 \times 10^5$ counts/min). TLC with cyclohexane–ethyl acetate (1 : 1) showed two peaks, one of which corresponded to 5β-pregnanediol and the other to 5β-hydroxypregnanolone. The mixture was separated by preparative TLC with the same system, giving $1{\cdot}26 \times 10^5$ counts/min of 5β-pregnanediol and $1{\cdot}45 \times 10^5$ counts/min of 5β-hydroxypregnanolone. One-half of the 5β-pregnanediol was then further purified by preparative TLC with $CH_2Cl_2$–methanol (97 : 3), giving $4{\cdot}70 \times 10^4$ counts/min. One-half of this material was diluted with 30 mg of carrier material, crystallized, acetylated, and again crystallized as shown in Table 1.

The 5β-hydroxypregnanolone zone was chromatographically homogeneous in $CH_2Cl_2$–methanol (97 : 3), $CH_2Cl_2$–acetone (9 : 1), and $CHCl_3$–ethyl acetate (4 : 1). A portion of this material, representing $1{\cdot}00 \times 10^5$ counts/min, was diluted with 1 mg of 5β-hydroxypregnanolone, dissolved in 0·2 ml acetone, cooled to 10°, and treated with 5μl of Kiliani's solution. After 10 min at 10°, the excess reagent was destroyed with a few drops of methanol, 0·2 ml water added, and the mixture extracted with four 1-ml portions of ethyl acetate. The extracts were washed with 0·5 ml of water, 0·5 ml of 10% $KHCO_3$, and two 0·5-ml portions of water, combined, and evaporated. TLC with $CH_2Cl_2$–acetone (9 : 1) showed a single radioactive product corresponding in mobility to 5β-hydroxypregnanedione. This material was then refluxed with 0·5 ml of acetic acid for 30 min, and the solvent was removed in vacuum. TLC of the product with the same system showed a single radioactive peak corresponding to progesterone. The remainder of the 5β-hydroxypregnanolone zone from above was diluted with 13·2 mg of carrier material, crystallized, acetylated, and again crystallized as shown in Table 1.

Fraction 4 (39 mg, $2{\cdot}97 \times 10^6$ counts/min) consisted mainly of 5α-pregnanedione, together with a small amount of the esterified material found in Fraction 3. Purification by preparative TLC with cyclohexane–ethyl acetate (1 : 1) and then with $CHCl_3$–ethyl acetate (19 : 1) gave chromatographically homogeneous 5α-pregnanedione ($9{\cdot}30 \times 10^5$ counts/min). This was diluted with 30 mg of carrier material, crystallized, reduced with $NaBH_4$ to 5α-pregnane-3β,20β-diol,[8] and again crystallized (Table 1).

Fraction 5 (10 mg, $1{\cdot}26 \times 10^6$ counts/min) was a complex mixture of several radioactive materials. Nine-tenths of it was chromatographed on a 20-g column of silica gel, packed into a chromatographic tube of 15 mm diameter as a slurry in $CHCl_3$–ethyl acetate (19 : 1). Fractions of 4 ml each were collected at 20 min intervals with $CHCl_3$–ethyl acetate (9 : 1). Fractions 16–24 ($1{\cdot}80 \times 10^5$ counts/min) contained a mixture of 5α-pregnanedione and 5β-pregnanedione. Separation by preparative TLC with $CHCl_3$–ethyl acetate (19 : 1) gave $5{\cdot}3 \times 10^4$ counts/min of 5α-pregnanedione and $2{\cdot}5 \times 10^4$ counts/min of 5β-pregnanedione. The latter was diluted with 33·6 mg of carrier 5β-pregnanedione, crystallized, reduced with $NaBH_4$ to 5β-pregnane-3α,20β-diol,[8] and again crystallized (Table 1).

Fractions 25–31 from the second column ($1{\cdot}96 \times 10^5$ counts/min) consisted mainly of progesterone. Fractions 32–37 ($3{\cdot}66 \times 10^5$ counts/min) were a mixture of progesterone and 5β-pregnanolone. Preparative TLC with $CH_2Cl_2$–methanol (23 : 2) gave $8{\cdot}00 \times 10^4$ counts/min of the latter. Fractions 38–40 ($1{\cdot}26 \times 10^5$ counts/min) showed a single peak corresponding to 5β-pregnanolone by TLC with $CH_2Cl_2$–methanol (97 : 3).

After acetylation, however, this material was resolvable by TLC with $CH_2Cl_2$–hexane (4 : 1, continuous development[16] for 4 hr) into two peaks, one corresponding to 5β-pregnanolone acetate and the other slightly less polar. Preparative TLC with the same system gave $5{\cdot}10 \times 10^4$ counts/min of the unknown acetate and $2{\cdot}30 \times 10^4$ counts/min of 5β-pregnanolone acetate. The latter was diluted with 28 mg of carrier material, crystallized, hydrolyzed with 0·1 N NaOH in methanol[8] to 5β-pregnanolone, and again crystallized (Table 1).

Fraction 6 (9 mg, $4{\cdot}20 \times 10^7$ counts/min) contained most of the radioactive progesterone, but TLC with $CH_2Cl_2$–methanol (97 : 3) indicated that some 5α-pregnanolone was also present. One-tenth of Fraction 6 was subjected to preparative TLC in the same system and the zone corresponding to 5α-pregnanolone isolated ($3{\cdot}12 \times 10^5$ counts/min). Pregnenolone cochromatographs with 5α-pregnanolone but can be separated by epoxidation.[8] When the radioactive material was epoxidized, TLC with $CH_2Cl_2$–methanol (97 : 3) showed no radioactivity corresponding to pregnenolone epoxide. The radioactive 5α-pregnanolone was then diluted with 30 mg of carrier material and acetylated. The acetate was purified by preparative TLC with $CH_2Cl_2$–methanol (99 : 1), crystallized, hydrolyzed as for 5β-pregnanolone acetate, and again crystallized (Table 1).

Fraction 7 (8 mg, $9{\cdot}27 \times 10^6$ counts/min) consisted almost entirely of radioactive progesterone. Fraction 8 (6 mg, $2{\cdot}14 \times 10^6$ counts/min) showed a single peak corresponding to 5β-hydroxypregnanolone by TLC with cyclohexane–ethyl acetate (1 : 1). With $CH_2Cl_2$–methanol (23 : 2), however, most of the radioactivity was shown to be associated with a less polar, unknown compound. One-tenth of Fraction 8 was subjected to TLC with the latter system and the zone corresponding to 5β-hydroxypregnanolone was isolated ($2{\cdot}0 \times 10^4$ counts/min). TLC with cyclohexane–ethyl acetate (1 : 1) showed a single radioactive peak corresponding to 5β-hydroxypregnanolone.

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[16] R. D. BENNETT and E. HEFTMANN, *J. Chromatog.* **21**, 488 (1966).