Tait, J. F., Little, B., Tait, S. A. S., and Flood, C. (1962), *J. Clin. Invest. 41,* 2093.

Tait, J. F., Tait, S. A. S., Little, B., and Laumas, K. R. (1961), *J. Clin. Invest. 40,* 72.

VandeWiele, R. L., MacDonald, P. C., Gurpide, E., and Lieberman, S. (1963), *Recent Progr. Hormone Res. 19,* 275.

Wallace, E. Z., and Lieberman, S. (1963), *J. Clin. Endocrinol. Metab. 23,* 90.

Warren, J. C., and Timberlake, C. E. (1962), *J. Clin. Endocrinol. Metab. 22,* 1148.

Wieland, R. G., Levy, R. P., Katz, D., and Hirschmann, H. (1963), *Biochim. Biophys. Acta 78,* 566.

Zimmermann, W. (1936), *Z. Physiol. Chem. 245,* 47.

Mechanisms of Steroid Oxidation by Microorganisms*

VI. Metabolism of 3β-Hydroxy-5α,6α-oxidoandrostan-17-one

S. S. LEE AND CHARLES J. SIH

From the School of Pharmacy, University of Wisconsin, Madison

Received April 10, 1964

When 3β-hydroxy-5α,6α-oxidoandrostan-17-one (IV) was exposed to *Nocardia restrictus,* 6α-hydroxyandrost-4-ene-3,17-dione (VI) and 3,6*S*-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (V), mp 145–147°, $[\alpha]_D^{25}$ +13°, λ_{max}^{alc} 280 mμ (ε, 2400), λ 3.01, 5.75, 5.84, 6.21, 6.32, and 6.70 μ (KBr) were obtained. 3β,5α,6β-Trihydroxyandrostan-17-one (IX) was transformed into 6β-hydroxyandrost-4-ene-3,17-dione (X) and 3,6*R*-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (XI), mp 98–100°, $[\alpha]_D^{25}$ +3°, λ_{max}^{alc} 280 mμ (ε, 2300), λ 2.98, 5.78, 5.84, 6.20, 6.31, and 6.70 μ (KBr) by the same organism. The absence of a 6α-hydroxylase in *N. restrictus* was established by isotopic experiments. The mechanism of epoxide opening appears to involve the oxidation of compound IV into 5α,6α-oxidoandrostane-3,17-dione (XII), which can undergo nonenzymatic rearrangement to yield compound VI. A convenient chemical procedure for the preparation of compound VI is described.

Our continued interest in defining the degradative pathway of androst-4-ene-3,17-dione (I) by *Nocardia restrictus* prompted us to search for more suitable methods for the preparation of substantial quantities of 9α-hydroxyandrost-4-ene-3,17-dione (II), a starting material for the synthesis of other degradative intermediates. Previous studies have shown that the initial degradative reactions of compound I by *N. restrictus* involve 9α-hydroxylation followed by 1,2-dehydrogenation or vice versa to yield 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (III) (Dodson and Muir, 1961). However, A-norandrost-4-ene-3,17-dione was converted into 9α-hydroxy-A-norandrost-4-ene-3,17-dione in good yields and the latter product was not further metabolized at a significant rate (Sih, 1962). On the basis of this result, it appeared that if one could prevent or delay the formation of a $\Delta^{1,4}$-dienone system in ring A, it might be possible to obtain 9α-hydroxysteroids in good yields. The readily available compound, 3β-hydroxy-5α,6α-oxidoandrostan-17-one (IV) was selected to test this hypothesis. Although no substantial quantities of the desired 9α-hydroxysteroid accumulated, we would like to report our observations on the metabolism of compound IV by *N. restrictus* and the mechanism of the opening of the epoxide in compound IV.

When compound IV was exposed to *N. restrictus* two products were formed. The first product, compound VI, was characterized as 6α-hydroxyandrost-4-ene-3,17-dione on the basis that its infrared spectrum was identical to an authentic sample and a mixed-melting-point determination gave no depression. The second product, compound V, was initially recognized as a phenol by virture of its ultraviolet spectrum λ_{max}^{alc} 280 mμ (ε, 2400). Its infrared spectrum showed bands at 3.01 μ (OH), 5.75 μ (5-membered ring ketone), 5.84 μ (6-membered ring ketone), 6.21, 6.32, and 6.70 μ (aromatic ring). Carbon-hydrogen analysis afforded figures consistent with $C_{19}H_{24}O_4$. Methylation of compound V with methyl iodide and potassium carbonate in acetone afforded its corresponding methyl ether, compound VII, whose NMR[1] spectrum showed bands at 9.02 τ (3 H, 18-Me); 7.75 τ (3 H, 19-Me); 6.23 τ (3 H, CH_3-O); 4.65 τ (1H, J's 8, 10 cps 6-H); 2.92, 3.05, 3.27 and 3.42 τ (two vicinal protons on aromatic ring); 2.75 τ (proton at C-4 on aromatic ring). Oxidation of compound VII with pyridine–chromic acid afforded 3-methoxy-9,10-secoandrosta-1,3,5(10)-triene-6,9,17-trione (VIII) on the basis of the following data: Carbon-hydrogen analysis was in good agreement with $C_{20}H_{24}O_4$; its ultraviolet spectrum showed bands at 237 mμ (ε, 7200) and 308 mμ (ε, 3100), characteristic of methoxyindanones; its infrared spectrum showed bands at 5.76, 5.85, 5.90, 6.22, 6.31, and 6.71 μ. As expected, compound VI was converted into V by exposure to *N. restrictus* via 9α-hydroxylation and 1,2-dehydrogenation. All these results suggest that the epoxide in compound IV had been opened and the structure of compound V is 3,6*S*-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (Cahn *et al.,* 1956).

Several examples of microbiological opening of steroid epoxides have been observed. Camerino *et al.* (1956) observed the conversion of 16α,17α-oxidopregn-4-ene-3,20-dione into 16α,20α-dihydroxy-17β-methyl-18-nor-17α-pregna-4,13-dien-3-one by yeast via a retropinacoline-type rearrangement. Camerino and Sciaky (1959) reported the transformation of 4β,5β-oxidopregnane-3,20-dione into 3β,4β,5α-trihydroxypregnan-20-one by yeast. Prochazka *et al.* (1961) noted that 3β-hydroxy-5,6-oxido-B-norandrostan-17-one was transformed into 3β,5α,6β-trihydroxy-B-nor-

---

* This investigation was supported in part by U. S. Public Health Service research grants (AM 04874 and AM 06110). For paper V of this series see Tsong *et al.* (1964).

[1] Abbreviation used in this work: NMR, nuclear magnetic resonance.

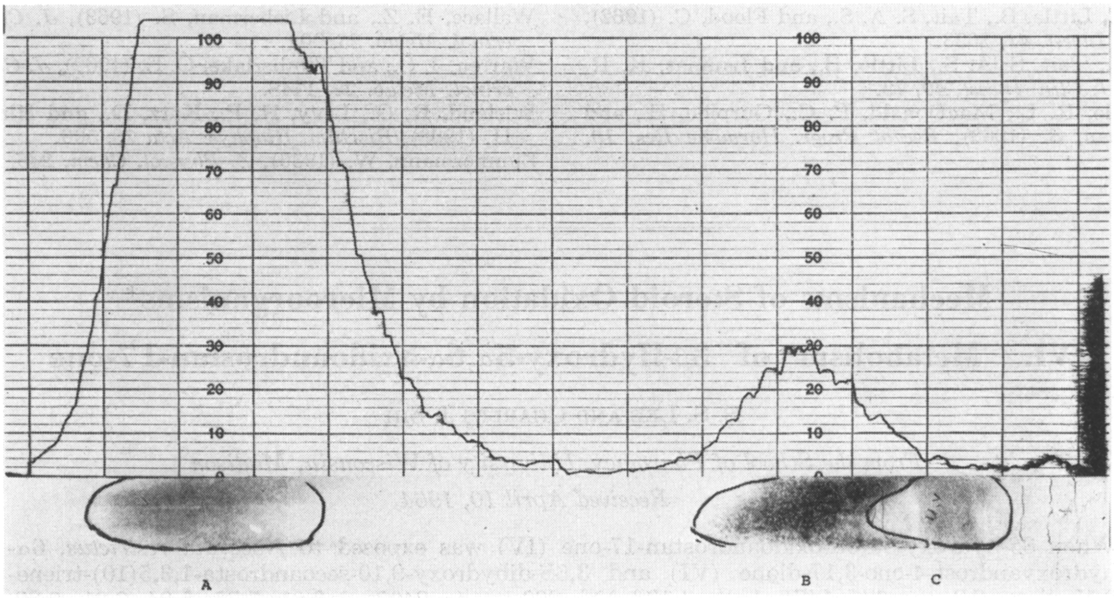
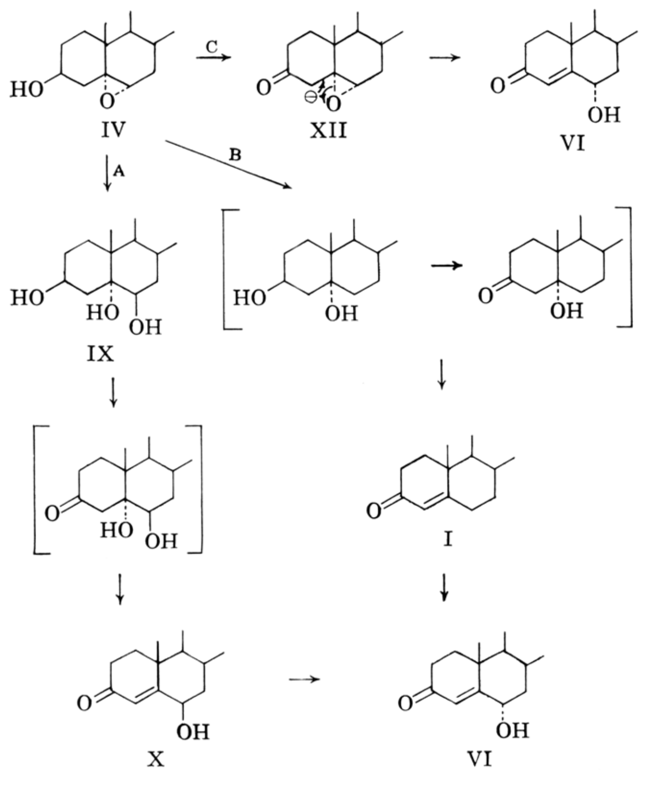


FIG. 1.—Detection of the possible presence of a 6α-hydroxylase. *N. restrictus* was grown in 25 ml of Difco nutrient broth in a 125-ml Erlenmeyer flask for 48 hours. Compound IV (12.5 mg in 0.25 ml of dimethylformamide) was then added to the flask. After 3.5 hours, 0.5 μc of androst-4-ene-3,17-dione-4-^{14}C in 0.05 ml of acetone was added to the flask along with 2.5 mg of nonradioactive androst-4-ene-3,17-dione, dissolved in 0.05 ml of dimethylformamide. After 5 hours the culture broth was extracted with chloroform and the chloroform extract was chromatographed on paper and developed in the toluene–propylene glycol system for 3 hours. After drying, the paper was sprayed with the Zimmerman reagent (Zimmermann, 1935) and the radioactivity was assayed with the radioactive autoscanner. A, androst-4-ene-3,17-dione; B, 9α-hydroxyandrost-4-ene-3,17-dione; C, 6α-hydroxyandrost-4-ene-3,17-dione.

androstan-17-one in low yields by *Rhizopus nigricans*. el-Tayeb *et al.* (1964) demonstrated the conversion of 16α,17α-oxidopregn-4-ene-3,20-dione into 16α-hydroxy-17a-oxaandrosta-1,4-diene-3,17-dione by *Cylindrocarpon radicicola*.

Since *N. restrictus* may possess a different mechanism of epoxide opening, a study was initiated to establish the mechanism of the conversion of compound IV into VI. Three possible mechanisms could be visualized and these could be represented as follows:



Mechanism A would involve the opening of the 5α,6α-oxide of compound IV into a transdiaxial glycol (IX) which parallels the acid-catalyzed opening of epoxides. Oxidation of the 3β-alcohol followed by spontaneous elimination (Sih *et al.*, 1962) would give 6β-hydroxyandrost-4-ene-3,17-dione (X); the latter compound could epimerize to yield compound VI. Mechanism B could proceed via a reductive type of opening to give an 5α-axial alcohol in a manner similar to that of metal hydrides. Oxidation of the 3β-alcohol followed by nonenzymatic elimination would yield androst-4-ene-3,17-dione (I); hydroxylation by an 6α-hydroxylase would lead to compound VI. Mechanism C would involve the oxidation of the 3β-alcohol to give the epoxy ketone (XII) which then rearranges to yield compound VI.

3β,5α,6β-Trihydroxyandrostan-17-one (IX) was prepared and incubated with *N. restrictus* to establish whether under our experimental conditions the 6β-alcohol could have epimerized. Two products were obtained after exposure of compound IX with this organism, one of which was identified as 6β-hydroxyandrost-4-ene-3,17-dione (X) on the basis that its infrared spectrum was identical to an authentic sample and no depression was noted in a mixed-melting-point determination. The other product was assigned the structure 3,6*R*-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (XI) on the basis of its ultraviolet spectrum $\lambda_{\max}^{\text{alc}}$ 280 mμ (ε, 2300); infrared spectrum 2.98, 5.78, 5.84, 6.20, 6.31, and 6.70 μ and carbon-hydrogen analysis. This experiment showed that there was no epimerization under our experimental conditions, making mechanism A an unlikely one. Mechanism B was eliminated on the basis of the following experiment. Radioactive androst-4-ene-3,17-dione-4-^{14}C was added to the fermentation medium and at various time intervals samples were withdrawn to determine whether any radioactivity resided in compound VI. Figure 1 shows that two major radioactivity peaks were obtained; these corresponded to androst-4-ene-3,17-dione (I) and 9α-hydroxyandrost-4-ene-3,17-dione (II). However, no significant quantities of radioactivity could be detected in compound VI. In a separate experiment, when nonradioactive androst-4-ene-3,17-dione was omitted as a carrier, no radioactivity could be detected on the paper chromatogram. This is consistent with the fact that compound II is rapidly metabolized by this organism in a series of reactions which eventually lead to CO_2 and H_2O (Sih, 1962); in contrast compound VI is only slowly metabolized by *N. restrictus*.

In order to establish whether pathway C is the mechanism of epoxide cleavage, $5\alpha,6\alpha$-oxidoandrost-4-ene-3,17-dione (XII) was prepared from compound IV by pyridine–chromic acid oxidation. Surprisingly, a substantial quantity of compound VI was obtained. However this observation is consistent with the results of other workers who have shown that 5,10-epoxy-3-ketone (Ruelas *et al.*, 1958), 9,11-epoxy-7-ketone (Djerassi *et al.*, 1953), and 5,6-epoxy-3-ketone (Campbell *et al.*, 1958) were capable of undergoing facile rearrangements in base to yield their corresponding unsaturated hydroxy ketone. Since one could not a priori predict the absence of an enzyme in *N. restrictus* also capable of catalyzing such a rearrangement, an experiment was designed to evaluate this possibility. Figure 2 shows that when compound XII was incubated with this organism, it was readily converted to compounds V and VI and 6α-hydroxyandrosta-1,4-diene-3,17-dione. However, autoclaved *N. restrictus* cells also transformed compound XII into VI, thereby establishing that the actual epoxide-opening reaction was indeed a nonenzymatic one. This mechanism is further confirmed by the fact that 17β-hydroxy-3-ethylenedioxy-$5\alpha,6\alpha$-oxidoandrostane was oxidized only to 3-ethylenedioxy-$5\alpha,6\alpha$-oxidoandrostan-17-one by the same organism.

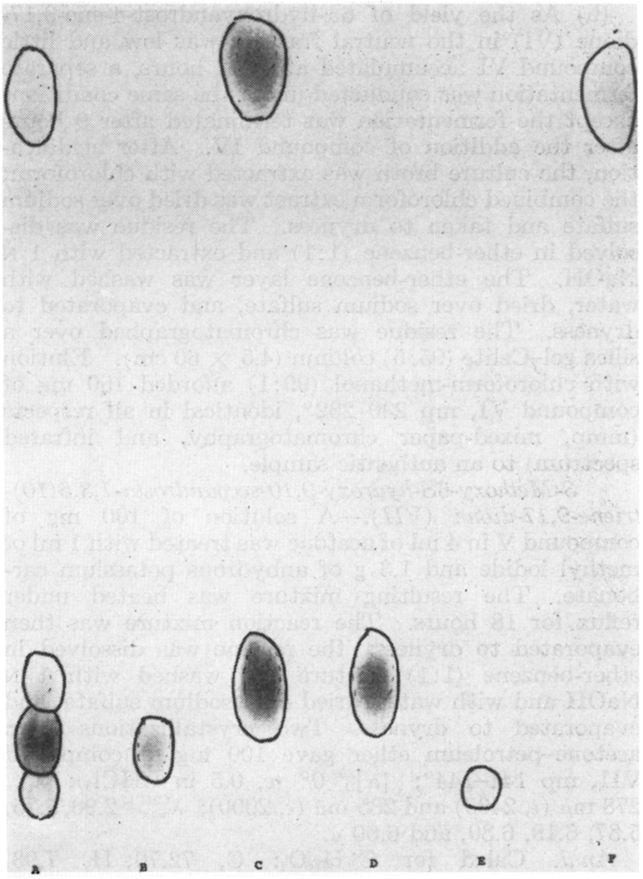


FIG. 2.—Reaction of $5\alpha,6\alpha$-oxidoandrostane-3,17-dione (XII) with intact and autoclaved cells of *N. restrictus*. *N. restrictus* was grown for 24 hours in 50 ml of Difco nutrient broth in a 250-ml Erlenmeyer flask. A duplicate flask of the 24-hour culture was autoclaved for 20 minutes and cooled. Compound XII (12 mg in 2 ml of dimethylformamide) was added to each flask and a sample was removed for analysis after 3 hours of incubation. An aliquot of the chloroform extract of the culture broth was spotted on Whatman No. 1 paper and developed for 3 hours in the toluene–propylene glycol system. The steroids were detected by spraying the paper with the Zimmermann reagent. A, compound XII + *N. restrictus*; B, 6α-hydroxyandrosta-1,4-diene-3,17-dione; C, compound XII + autoclaved *N. restrictus*; D, 6α-hydroxyandrost-4-ene-3,17-dione; E, 3,6*S*-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione; F, $5\alpha,6\alpha$-oxidoandrostane-3,17-dione (XII).

EXPERIMENTAL

Melting points, determined on a Thomas-Hoover melting-point apparatus, are corrected. Ultraviolet-absorption spectra were determined on a Cary Model 11 MS recording spectrophotometer and 96% ethanol was used as solvent. Infrared spectra were recorded on a Beckman IR 5A double-beam infrared-recording spectrophotometer. Microanalyses were carried out by Mr. J. Alicino of Metuchen, N.J. The NMR spectrum was determined on a Varian Associates recording spectrometer (A60) at 60 mc in deuterated chloroform with tetramethylsilane as an internal standard. Chemical shifts are reported in τ values (ppm) (Tiers, 1958). Radioactivity on paper-chromatographic strips was measured on a Vanguard Autoscanner Model 880. The paper-chromatographic system used throughout this work consisted of toluene–propylene glycol (Zaffaroni *et al.*, 1950). "Petroleum ether" refers to the fraction of bp 60–80°. Silica gel (Mallinkrodt 2847) was washed with acetone-ether (2:1) and dried at 90–100°. Radioactive androst-4-ene-3,17-dione-4-^{14}C (specific activity = 22.3 mc/mmole) was purchased from The New England Nuclear Corp.

Fermentation of 3β-Hydroxy-$5\alpha,6\alpha$-oxidoandrostan-17-one (IV).—(a) *Nocardia restrictus* No. 545 (Wang and Sih, 1963) was grown in 10.8 liters of Difco nutrient broth (twenty-seven 2-liter Erlenmeyer flasks) at 25° on a rotary shaker. After 24 hours of incubation, 10.8 g of compound IV, dissolved in 85 ml of dimethylformamide, was distributed equally among the flasks. After 12 hours the culture broth was acidified with glacial acetic acid to a *p*H of 2.0 and was extracted three times with three 3-liter portions of chloroform. The combined chloroform extract was dried over sodium sulfate and evaporated to dryness. An aliquot of the chloroform extract was spotted on Whatman No. 1 paper and developed for 3 hours in the toluene–propylene glycol system. Two spots were noted under the ultraviolet scanner whose R_F values were 0.13 and 0.03. The residue was dissolved in 300 ml of a mixture of ether and benzene (1:1) and extracted three times with 100-ml portions of 1 N NaOH. The aqueous-alkaline fraction was acidified with acetic acid and extracted with three 100-ml portions of chloroform. The combined chloroform extract, after washing with water, was dried over sodium sulfate and evaporated to dryness. Two crystallizations from acetone–petroleum ether gave 1.0 g of 3,6*S*-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (V), mp 145–147°; $[\alpha]_D^{24}$ +13° (*c*, 0.5 in $CHCl_3$); λ_{max}^{alc} 280 mμ (ϵ,2400); λ_{max}^{KBr} 3.01, 5.75, 5.84, 7.21, 6.32, and 6.70 μ.

Anal. Calcd for $C_{19}H_{24}O_4 \cdot {}^1/_2H_2O$: C, 70.13; H, 7.74. Found: C, 70.04, 69.89; H, 8.07, 7.99.

(b) As the yield of 6α-hydroxyandrost-4-ene-3,17-dione (VI) in the neutral fraction was low and little compound VI accumulated after 12 hours, a separate fermentation was conducted under the same conditions except the fermentation was terminated after 9 hours after the addition of compound IV. After acidification, the culture broth was extracted with chloroform; the combined chloroform extract was dried over sodium sulfate and taken to dryness. The residue was dissolved in ether-benzene (1:1) and extracted with 1 N NaOH. The ether-benzene layer was washed with water, dried over sodium sulfate, and evaporated to dryness. The residue was chromatographed over a silica gel–Celite (95:5) column (4.5 × 60 cm). Elution with chloroform-methanol (99:1) afforded 150 mg of compound VI, mp 230–232°, identical in all respects (mmp, mixed-paper chromatography, and infrared spectrum) to an authentic sample.

3-Methoxy-6S-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (VII).—A solution of 100 mg of compound V in 4 ml of acetone was treated with 1 ml of methyl iodide and 1.3 g of anhydrous potassium carbonate. The resulting mixture was heated under reflux for 18 hours. The reaction mixture was then evaporated to dryness; the residue was dissolved in ether-benzene (1:1) mixture and washed with 1 N NaOH and with water, dried over sodium sulfate, and evaporated to dryness. Two crystallizations from acetone–petroleum ether gave 100 mg of compound VII, mp 141–144°; $[\alpha]_D^{25}$ 0° (*c*, 0.5 in $CHCl_3$); λ_{max}^{alc} 278 mμ (ϵ, 2400) and 285 mμ (ϵ, 2000); $\lambda_{max}^{CHCl_3}$ 2.90, 5.75, 5.87, 6.19, 6.30, and 6.69 μ.

Anal. Calcd for $C_{20}H_{26}O_4$: C, 72.70; H, 7.93. Found: C, 72.33; H, 8.26.

3-Methoxy-9,10-secoandrosta-1,3,5(10)-triene-6,9,17-trione(VIII).—To 160 mg of compound VII in 26 ml of pyridine was added 150 mg of chromic trioxide and the reaction mixture was left at room temperature for 24 hours. After the excess chromic acid was destroyed with ethanol, the mixture was diluted with water and extracted with an ether-benzene (1:1) mixture. The combined ether-benzene layer was dried over sodium sulfate and evaporated to dryness. The residue was chromatographed over a cellulose-powder column (Sih and Bennett, 1960) (2.5 × 45 cm) using propylene glycol as the stationary phase. Elution of the column with isooctane saturated with propylene glycol afforded 120 mg of compound VIII (recrystallized from acetone–petroleum ether); mp 121–122°; $[\alpha]_D^{25}$ +42° (*c*, 0.7 in $CHCl_3$); λ_{max}^{alc} 237 mμ (ϵ, 7200) and 308 mμ (ϵ, 3100); λ_{max}^{KBr} 5.76, 5.85, 5.90, 6.22, 6.31, and 6.71 μ.

Anal. Calcd for $C_{20}H_{24}O_4$: C, 73.14; H, 7.37. Found: C, 73.46; H, 7.56.

Fermentation of 3β,5α,6β-Trihydroxyandrostan-17-one (IX).—(a) 10.8 g of compound IX was fermented under conditions identical to those previously described. After 13 hours the culture broth was acidified with glacial acetic acid and extracted three times with 3-liter portions of chloroform. The combined chloroform extract was dried over sodium sulfate and taken to dryness. An aliquot of the chloroform extract was spotted on Whatman No. 1 paper and developed for 3 hours in the toluene–propylene glycol system. Two spots were observed under the ultraviolet scanner. The R_F values were 0.33 and 0.03. The residue was dissolved in ether-benzene (1:1) and extracted with 1 N NaOH to remove the phenolic steroids. The benezene-ether layer was washed with water, dried over sodium sulfate, and evaporated to dryness. The residue was chromatographed over a cellulose-powder column (4 × 60 cm). Propylene glycol was used as the stationary phase. Elution of the column with benzene saturated with propylene glycol gave 110 mg of 6β-hydroxyandrost-4-ene-3,17-dione (X) (recrystallized from acetone–petroleum ether; mp 190–192°), identical in all respects (mmp and infrared spectrum) to an authentic sample of compound X.

(b) The product in the phenolic fraction (R_F 0.03) decomposed on standing in alkali to give two additional phenolic compounds. Therefore a separate fermentation was conducted and no alkali was used in the preparation. *N. restrictus* was grown in 10.8 liters of Difco nutrient broth (twenty-seven 2-liter Erlenmeyer flasks) at 25° on a rotary shaker. After 24 hours of incubation, 6.5 g of compound IX, dissolved in 70 ml of dimethylformamide, was distributed equally among the flasks. After 12 hours the culture broth was extracted with three 3-liter portions of chloroform. The combined chloroform extract was dried over sodium sulfate and evaporated to dryness. The residue was triturated several times with acetone to remove the acetone-insoluble residual substrate. The acetone extract was chromatographed over a cellulose-powder column (3 × 50 cm). Propylene glycol was used as the stationary phase. The mobile phase initially consisted of cyclohexane-benzene (1:1) and 10-ml fractions were collected. After fraction 120 the mobile phase was changed to benzene. Fractions 193–218 were pooled and evaporated to dryness. Two crystallizations from acetone–petroleum ether afforded 30 mg of compound XI, mp 98–100°; $[\alpha]_D^{25}$ +3° (*c*, 0.6 in $CHCl_3$); ${}_{max}^{alc}\lambda$ 280 mμ (ϵ, 2300); λ_{max}^{KBr} 2.98, 5.78, 5.84, 6.20, 6.31, and 6.70 μ.

Anal. Calcd for $C_{19}H_{24}O_4$: C, 72.12; H, 7.65. Found: C, 71.93; H, 7.83.

Oxidation of 3β-Hydroxy-5α,6α-oxidoandrostan-17-one (IV).—To 8 g of compound IV in 80 ml of pyridine was added 8 g of chromic acid, dissolved in 80 ml of pyridine. The mixture was left standing at room temperature for 3 hours and was then poured into 500 ml of ether. The ethereal layer was washed with acetic acid, sodium carbonate, and water. It was then dried over sodium sulfate and the solvent was removed to yield 7.3 g of residue. Three g of this residue was chromatographed over a cellulose-powder column (4 × 60 cm) using propylene glycol as the stationary phase. Elution of the column with cyclohexane saturated with propylene glycol afforded 0.35 g of 5α,6α-oxidoandrostane-3,17-dione (XII) after two crystallizations from acetone–petroleum ether, mp 190–192°; $[\alpha]_D^{25}$ +8° (*c*, 0.9 in $CHCl_3$); $\lambda_{max}^{CHCl_3}$ 5.75 and 5.85 μ; ultraviolet absorption at 238 mμ was (ϵ, 75). Paper chromatography showed a single spot in both the isooctane–propylene glycol and toluene–propylene glycol systems. Reported: mp 195–201°; $[\alpha]_D$ −2° ($CHCl_3$) (Campbell *et al.*, 1958).

Anal. Calcd for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67. Found: C, 75.07; H, 8.96. Further elution of the column with benzene saturated with propylene glycol gave 2.05 g of compound IV, mp 225–227° and 0.50 g of compound VI, mp 230–232°.

ACKNOWLEDGMENTS

The authors wish to thank Dr. M. Ehrenstein for a sample of 6α-hydroxyandrost-4-ene-3,17-dione and Dr. G. S. Fonken for a sample of 6β-hydroxyandrost-4-ene-3,17-dione.

REFERENCES

Cahn, R. S., Ingold, C. K., and Prelog, V. (1956), *Experientia 12*, 81.

Camerino, B., Modelli, R., and Spalla, C. (1956), *Gazz. Chim. Ital. 86*, 1219.

Camerino, B., and Sciaky, R. (1959), *Gazz. Chim. Ital. 89*, 654.

Campbell, J. A., Babcock, J. C., and Hogg, J. A. (1958), *J. Am. Chem. Soc. 80*, 4717.

Djerassi, C., Mancera, O., Romo, J., and Rosenkranz, G. (1953), *J. Am. Chem. Soc. 75*, 3505.

Dodson, R. M., and Muir, R. D. (1961), *J. Am. Chem. Soc. 83*, 4627.

el-Tayeb, O., Knight, S. G., and Sih, C. J. (1964), *Biochim. Biophys. Acta* (in press).

Prochazka, Z., Fajkos, J., Joska, J., and Sorm, F. (1961), *Coll. Czech. Chem. Commun. 26*, 2068.

Ruelas, J. P., Iriarte, J., Kincl, F., and Djerassi, C. (1958), *J. Org. Chem. 23*, 1744.

Sih, C. J. (1962), *Biochim. Biophys. Acta 62*, 541.

Sih, C. J., and Bennett, R. E. (1960), *Biochim. Biophys. Acta 38*, 378.

Sih, C. J., Kupchan, S. M., el-Tayeb, O., and Afonso, A. (1962), *J. Med. Pharm. Chem. 5*, 629.

Tiers, G. V. D. (1958), *J. Phys. Chem. 62*, 1151.

Tsong, Y. Y., Wang, K. C., and Sih, C. J. (1964), *Biochim. Biophys. Acta* (in press).

Wang, K. C., and Sih, C. J. (1963), *Biochemistry 2*, 1238.

Zaffaroni, A., Burton, R. B., and Keutman, E. H. (1950), *Science 111*, 6.

Zimmermann, W. (1935), *Z. Physiol. 233*, 257.

Lipid Components of Leaves. V. Galactolipids, Cerebrosides, and Lecithin of Runner-Bean Leaves*

P. S. SASTRY† AND M. KATES

From the Division of Biosciences,
National Research Council, Ottawa, Canada

Received April 15, 1964

Mono- and digalactosyl diglycerides have been isolated from runner-bean leaves and shown to have the structures 2,3-di-*O*-linolenoyl-1-*O*-β-D-galactopyranosyl-D-glycerol (I), and 2,3-di-*O*-linolenoyl-1-*O*-β-(6-*O*-α-D-galactopyranosyl-D-galactopyranosyl)-D-glycerol (II), respectively. A cerebroside fraction has also been isolated and found to contain glucose as sole sugar constituent; α-hydroxy acids (chiefly α-hydroxypalmitic, α-hydroxy-C_{22}, and α-hydroxy-C_{24} acids) were the only fatty acids present, and the long-chain bases consisted chiefly of C_{18}-dehydrophytosphingosine with small amounts of C_{18}-phytosphingosine, C_{18}-dihydrosphingosine, and an isomer of C_{18}-sphingosine. The lecithin fraction was isolated in pure form and found to contain palmitic (27%), stearic (6%), oleic (4%), linoleic (38%), and linolenic (26%) acids; almost all the saturated acids were found to be in the α′ position.

The lipids of leaves are known to consist of more than a score of components. These include neutral lipids such as hydrocarbons, waxes, alcohols, quinones, sterols, sterol esters, and glycerides (Zill and Harmon, 1962; Weenink, 1962; Nichols, 1963; Lepage, 1964); phospholipids, such as lecithin, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol and polyglycerol phosphatides (Benson and Maruo, 1958; Benson and Strickland, 1960; Wintermans, 1960; Kates, 1960; Wheeldon, 1960; Nichols, 1963); glycolipids such as mono- and digalactosyl diglycerides (Benson *et al.*, 1958, 1959a; Wintermans, 1960; Kates, 1960; Weenink, 1961; Zill and Harmon, 1962) and sterol glycosides (Nichols, 1963); and a sulfolipid (Benson *et al.*, 1959b; O'Brien and Benson, 1964).

Previous attempts in this laboratory to separate the lipids of runner-bean leaves by chromatography on silicic acid columns did not result in clear-cut resolutions of the components (Kates and Eberhardt, 1957). Four fractions were obtained, the main components of which were: fraction I, glycolipids (including phosphatidylglycerol and sulfolipid); fraction II, phosphatidylethanolamine and phosphatidylserine; fraction III, an amino acid–containing phosphatide; and fraction IV, lecithin. Subsequently, evidence for the presence of at least four glycolipids, designated as a, b, c, and d, was obtained by chromatography on silicic acid–impregnated paper (Kates, 1960). Zill and Harmon (1962) found evidence for four similar glycolipids in spinach leaves.

Three of these glycolipid components have now been isolated from fraction I and shown to be monogalactosyl diglyceride (glycolipid d), digalactosyl diglyceride (glycolipid a), and glucocerebrosides (glycolipid b); the lecithin in fraction IV has also been isolated and characterized. The isolation procedures and structure determinations are described here in detail.

MATERIALS AND METHODS

Extraction of Lipids.—Scarlet runner-bean (*Phaseolus multiflorus*) plants were grown in the greenhouse and harvested after 20–28 days of growth (Kates and Eberhardt, 1957). One kg of fresh primary leaves were extracted in 100-g batches with isopropanol followed by chloroform, as described previously (Kates and Eberhardt, 1957). The lipid material thus obtained weighed 9.38 g (9.5% of dry wt) and contained 82.2 mg of phosphorus; it was stored in chloroform solution at −10°.

Column Chromatography.—Total leaf lipids were chromatographed on columns of silicic acid–Celite (4:1, w/w) as described previously (Kates and Eberhardt, 1957). The glycolipid fraction obtained (fraction I, Table I) was rechromatographed on columns of silicic acid (Bio-Rad Laboratories, −325 mesh) made up in methanol and prewashed with 3 column volumes of chloroform; separation of glycolipid d from glycolipids a and b was thereby achieved. Columns of silicic acid (Bio-Rad Laboratories) prepared accord-

---

* Issued as N.R.C. No. 8126. Preliminary reports of this work have appeared previously (Sastry and Kates, 1963, 1964).

† N.R.C. Postdoctoral Fellow, 1961–63. Present address: Department of Physiological Chemistry, University of Wisconsin, Madison, Wisc.