uppermost zone the composition was: zone 1, 9.0%; zone 2, 19.2%; zone 3, 29.5%; zone 4, 39.8%; and zone 5, 2.5%.

Zone 2 Sterol and Derivatives.—The azoyl ester was crystallized from benzene–ethanol and hydrolyzed. The acetate was prepared and crystallized once from aq. ethanol. The acetate contained 0.3% of provitamin D as calculated from the ultraviolet spectrum. This was removed with maleic anhydride as previously described. After several crystallizations from aq. ethanol, the acetate melted at 140°C., $[\alpha]^{22}D + 6.4°$ (c 3.2 in $CHCl_3$).

Anal. Calcd. for $C_{30}H_{48}O_2$: C, 81.76; H, 10.98. Found: C, 81.80; H, 10.66.

The acetate was hydrolyzed and the free sterol crystallized from aq. ethanol, m.p. 131°, $[\alpha]^{22}D$ +6.0° (c 2.0 in $CHCl_3$).

Anal. Calcd. for $C_{28}H_{46}O$: C, 84.35; H, 11.63. Found: C, 84.28; H, 11.82.

The benzoate was crystallized from acetone, m.p. 160°, $[\alpha]^{22}D$ +11.9° (c 1.4 in $CHCl_3$); molecular rotational differences: 7,24-ergostadien-3β-ol, Δ^{Ac} +4, Δ^{Bz} + 34; Δ^7-stenols, Δ^{Ac} −15 ± 15, Δ^{Bz} +20 ± 14.[20]

Anal. Calcd. for $C_{34}H_{48}O_2$: C, 83.55; H, 9.90. Found: C, 83.64; H, 9.86.

Saponification Equivalent.—Zone 2 acetate, 257.766 mg., was saponified in 5.00 ml. of 0.04736 N alcoholic NaOH for 1 hour and the residual alkali was back-titrated with 17.95 ml. of 0.09968 N HCl. A correction factor, 0.9935, was employed which was derived from several determinations of the molecular weight of highly purified cholesteryl acetate.

Anal. Calcd. for $C_{30}H_{48}O_2$: mol. wt., 440.7. Found: mol. wt., 442.6.

Perbenzoic Acid Titration.—On standing for five days at −5° in an excess of perbenzoic acid in $CHCl_3$, 19.006 mg. of zone 2 sterol consumed 2.11 mg. of oxygen. The theoretical uptake for three atoms of oxygen is 2.07 mg.

Ozonolysis.—Zone 1 sterol (100 mg.) was suspended in 4 ml. of acetaldehyde-free acetic anhydride–acetic acid (4:1) and cooled in an ice-bath. Ozonated oxygen was passed through the suspension at a rate of 10 mg. ozone/l. of oxygen until all of the solid was dissolved. Water and zinc dust were then added and the mixture was heated in a water-bath in order to decompose the ozonide. The mixture was steam distilled into a 0.5% dimedone solution. The pH of the solution was adjusted to 5.8 and the precipitate collected.

The dimedone derivative weighed 52.8 mg. (71.8% of the theory for one methylene group), recrystallized m.p. 189°, mixed m.p. 190° with authentic formaldehyde dimedone.

Anal. Calcd. for $C_{17}H_{24}O_4$: C, 69.82; H, 8.27. Found: C, 69.91; H, 8.49.

The filtrate from the dimedone solution was steam distilled into a 2,4-dinitrophenylhydrazone solution, which was then extracted with ethyl acetate. No hydrazone was obtained.

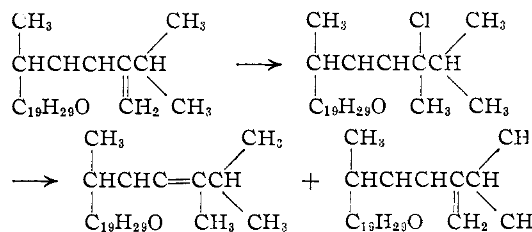


Fig. 1.

Rearrangement with HCl.—Zone 2 acetate (430 mg.) was dissolved in 5 ml. of $CHCl_3$ and HCl gas was led through the solution for 6 hours. The solvent was evaporated and the residue was boiled for 10 hours with 6 ml. of acetic anhydride. The solution was then diluted with water, the precipitate collected and chromatographed on an alumina column, from which 380 mg. of material was recovered.

The product from the column was suspended in 10 ml. of acetic anhydride–acetic acid (4:1) and ozonized as described above. The dimedone derivative weighed 71 mg. (28.3% calculated as acetate), m.p. 189° after crystallization, mixed m.p. 189° with authentic formaldehyde dimedone.

Anal. Calcd. for $C_{17}H_{24}O_4$: C, 69.82; H, 8.27. Found: C, 69.85; H, 8.89.

The remaining dimedone solution was steam distilled into a 2,4-dinitrophenylhydrazone solution. The solution was extracted with ethyl acetate and the evaporation residue chromatographed on a silicic acid–Celite column (2:1). Skellysolve C–benzene (2:1) moved a band which was eluted (66 mg. representing 28.7% calculated as acetate) and crystallized from aq. methanol, m.p. 123°, mixed m.p. 123° with authentic methyl isopropyl ketone.

Anal. Calcd. for $C_{11}H_{14}O_4N_4$: C, 49.62; H, 5.30. Calcd. for $C_{12}H_{16}O_4N_4$: C, 51.42; H, 5.75. Found: C, 49.68; H, 5.26.

The 2,4-dinitrophenylhydrazone of the ozonolysis product and of methyl isopropyl ketone had identical R_f values on a descending chromatogram run for 2.5 days in isopropyl alcohol–water (1:1) on Whatman No. 1 filter paper.[23] Ethyl isopropyl ketone, isovaleraldehyde, methyl isopropyl acetaldehyde and ethyl isopropyl acetaldehyde had lower R_f values.

Acknowledgment.—Mr. A. P. Ronald of our microanalytical section performed many of the analyses and recorded the infrared spectra.

(23) R. B. Seligman and M. D. Edmonds, *Chemistry & Industry*, 1406 (1955).

VANCOUVER 2, B. C., CANADA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA]

The Biosynthesis of Ergosterol: Its Relationship to the Squalene Hypothesis[1,2]

BY WILLIAM G. DAUBEN, THOMAS W. HUTTON AND GEORGE A. BOSWELL[3]

RECEIVED JULY 7, 1958

C^{14}-Ergosterol was biosynthesized from 1-C^{14}-acetate and the distribution of the labeled atoms studied. By conversion of ergosterol to progesterone it was shown that the distribution of label between the side-chain and the nucleus was that predicted on the basis of the squalene hypothesis. The specific carbons, C-3, C-4, C-11 and C-12 were obtained by degradation of appropriate precursors, and it was found that C-4, C-11 and C-12 were derived from the carboxyl of acetate, again as predicted by the squalene hypothesis. These results strongly support the concept of the utilization of the intact acyclic triterpene, squalene, in the biosynthesis of all steroids.

The squalene precursor[4] hypothesis for the mechanism of biosynthesis of steroids and tetracyclic triterpenes (C-30 steroids) has been widely investigated and the results obtained have substantiated

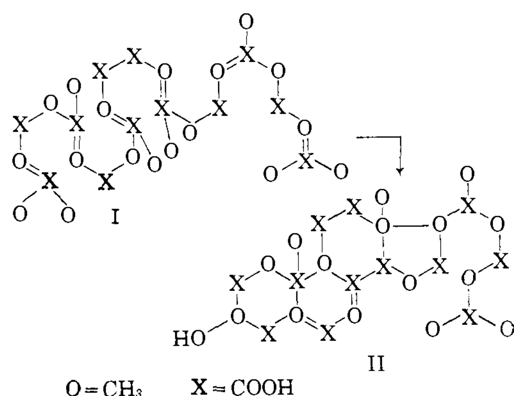
(1) This work was supported, in part, by Grant No. AT (11-1), Project No. 16, U. S. Atomic Energy Commission.

(2) A preliminary communication of a portion of this work has appeared in THIS JOURNAL, **78**, 2647 (1956).

(3) National Science Foundation Predoctoral Fellow, 1956–1957.

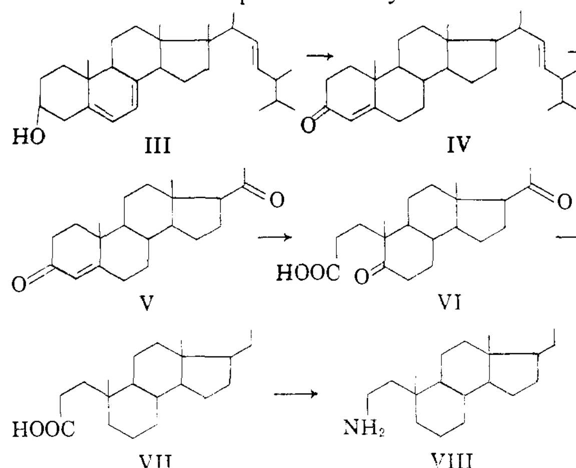
(4) R. B. Woodward and K. Bloch, THIS JOURNAL, **75**, 2013 (1953); W. G. Dauben, S. Abraham, S. Hotta, I. L. Chaikoff, H. L. Bradlow and A. H. Soloway, *ibid.*, **75**, 3038 (1953); A. Eschenmoser, L. Ruzicka, O. Jeger and D. Arigoni, *Helv. Chim. Acta*, **38**, 1890 (1955).

the concept.[4-6] This concept requires that intact squalene be a direct precursor of the tetracyclic ring system and not simply an efficient source of a substance of modified structure which closes to the cyclic ring system. If intact squalene is, indeed, the direct precursor, the symmetry feature of this acyclic triterpene presents a demanding requirement as to the source of various carbon atoms of the tetracyclic nucleus. For example, the two central carbon atoms of squalene (I) are derived from the carboxyl of acetate, and it is only in this position that two carbons in a juxtaposition are derived from the same progenitor.[7] These two central carbon atoms are the potential C-11 and C-12 of a steroid (II), and if squalene is cyclized as indicated in the hypothesis, these two carbon atoms in the steroid also must be derived from the carboxyl of acetate. In order to locate this point of biosynthetic symmetry, ring C of the steroid must be degraded.



The sterol chosen for study was ergosterol (III) since the presence of a homoannular diene in ring B facilitates the preparation of compounds required for the degradation of ring C. Furthermore, this sterol being a C-28 sterol derived from yeast also would serve to illustrate the generality of the squalene hypothesis. Prior to this study, three groups of investigators have examined the biosynthesis of ergosterol. In 1951, Ottke, Tatum, Zabin and Bloch[8] using an "acetate-less" mutant of *Neurospora crassa* found that the ergosterol obtained when a double labeled acetate was used as the carbon source could have derived at least 26 of its 28 carbon atoms from acetate. In 1953, Hanahan and Wakil[9] biosynthesized ergosterol by allowing a "Co-A" enriched *Saccharomyces cerevisiae* to grow in the presence of 1-C^{14}-acetate. By partial degradation of the side-chain of the steroid it was found that C-23 and C-25 were derived from the carboxyl of acetate and were equally labeled as predicted by the squalene hypothesis. The specific activity of these side-chain carbon atoms, however, was found to to be 40% lower than expected. These authors concluded that a greater amount of labeling had occurred in the formation of the ergosterol nucleus than in the formation of the side-chain, a conclusion which would suggest that ergosterol could not arise through a cyclization of intact squalene. Finally, in 1956, Corwin, Schroeder and McCullough[10] employing whole yeast and a cell-free system derived from it, obtained results which they interpreted to suggest that squalene was not an obligatory intermediate in the biosynthesis of yeast sterols. Recently, Loud and Bucher[11] have demonstrated the presence of two distinct squalene pool in mammalian systems and the presence of a similar situation in yeast would permit an explanation of the results of Corwin and his co-workers which would be in harmony with the squalene hypothesis.

In view of the conflicting nature of the work on the biosynthesis of ergosterol as far as the squalene hypothesis was concerned, it was necessary to re-evaluate the role of squalene in the process, in general, before utilizing a yeast sterol for a study of the progenitor of C-11 and C-12 in ring C of a sterol. This was accomplished by comparing, again, the specific activities of the side-chain and the nucleus and comparing the specific activity of specific carbon atoms in ring A with that expected on the basis of the squalene theory.



Turning attention first to the side-chain removal, C^{14}-ergosterol (III), obtained from the "acetate-less" mutant of *Neurospora crassa*[12] grown in the presence of 1-C^{14}-acetate, was converted to progesterone (V) *via* 4,22-ergostadien-3-one (IV) by the excellent procedure developed by the workers at the Upjohn Company.[13] As shown in Table I, the specific activity of IV and V was that predicted on the basis of the squalene hypothesis,[14] thus estab-

(5) J. A. Olsen, Jr., M. Lindberg and K. Bloch, *J. Biol. Chem.*, **226**, 941 (1957), and earlier papers; W. G. Dauben, Y. Ban and J. H. Richards, THIS JOURNAL, **79**, 968 (1957), and earlier papers; J. W. Cornforth, I. Y. Gore and G. Popjak, *Biochem. J.*, **65**, 94 (1957), and earlier papers.

(6) For reviews see, J. W. Cornforth, *Revs. Pure Appl. Chem.*, **4**, 286 (1954); G. Popjak, *Roy. Inst. Chem.*, Lecture No. 2 (1955); K. Bloch in "Currents in Biochemistry," Edited by D. Green, Interscience Publishers, Inc., New York, N. Y., 1956.

(7) J. W. Cornforth and G. Popjak, *Biochem. J.*, **58**, 403 (1954).

(8) R. C. Ottke, E. L. Tatum, I. Zabin and K. Bloch, *J. Biol. Chem.*, **189**, 429 (1951).

(9) D. J. Hanahan and S. J. Wakil, THIS JOURNAL, **75**, 272 (1953).

(10) L. M. Corwin, L. J. Schroeder and W. G. McCullough, *ibid.*, **78**, 1372 (1956).

(11) A. V. Loud and N. L. R. Bucher, *J. Biol. Chem.*, **233**, 37 (1958).

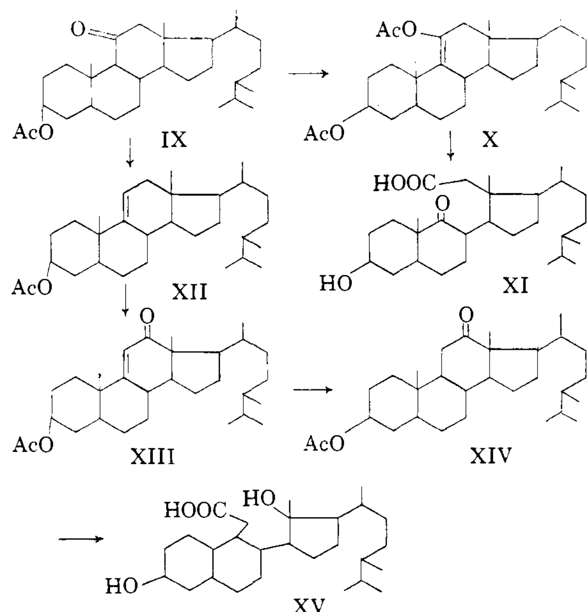
(12) We are indebted to Professor E. A. Adelberg, Department of Bacteriology, University of California, for the inoculum.

(13) D. A. Shephard, R. A. Donia, J. A. Campbell, B. A. Johnson, R. P. Holysz, S. Slomp, Jr., J. E. Stafford, R. L. Pederson and A. C. Ott, THIS JOURNAL, **77**, 1212 (1955).

(14) The calculated activities allow for the fact that C-28 is not derived from acetate, see H. Danielsson and K. Bloch, *ibid.*, **79**, 500 (1957); W. G. Dauben, G. J. Fonken and G. A. Boswell, *ibid.*, **79**, 1000 (1957); and G. J. Alexander and E. Schwenk, *ibid.*, **79**, 4554 (1957).

lishing that there was no greater specificity in acetate utilization for the nucleus over the side-chain.

To obtain individual carbon atoms, progesterone (V) was ozonized and the 5,20-diketo-3,5-seco-A-norpregnan-3-oic acid (VI) was obtained. As has been shown earlier,[15] C-4 was isolated as formic acid



which, in turn, was oxidized to carbon dioxide with mercuric acetate. Wolff–Kishner reduction of the diketo-seco-acid VI afforded 3,5-seco-A-norpregnan-3-oic acid (VII). This acid was decarboxylated by reaction with sodium azide in sulfuric acid and the carbon dioxide generated which corresponded to C-3 in the original ergosterol was collected. The resulting 3,4-bisnor-2,5-seco-2-aminopregnane (VIII)

TABLE I

DISTRIBUTION OF C^{14} IN ERGOSTEROL BIOSYNTHESIZED FROM 1-C^{14}-ACETATE

Compound	Specific activity (dis./min./mg. $BaCO_3$) Found	Calcd.[a]
IV	69.5[b]	..
V (before dilution)	76.5[b]	77.5[b]
V (after dilution)	14.3	..
VI	13.0	13.5
$BaCO_3$ from C-4	29.5	29.5
VIII (as acetyl der.)	12.5	12.5
$BaCO_3$ from C-3	0	0
IX	7.5[c]	..
$BaCO_3$ from C-11	13.5	17.5[c]
$BaCO_3$ from C-12	13.0	17.5[c]

[a] Based upon the squalene hypothesis which calls for 15 carbons derived from the methyl of acetate and 12 carbons from the carboxyl of acetate and 1 carbon from another source. [b] Cts./min./mg. $BaCO_3$. [c] The error in the determination of the specific activity is ~20% since the total number of disintegrations in chamber was at the lower limit of detection.

was isolated as its acetyl derivative. As shown in Table I, C-4 of ring A which should be derived from the carboxyl of acetate was labeled while C-3, derived from the methyl of acetate, was devoid of radioactivity. Furthermore, the specific activities of C-4, VI and VIII were those predicted on the basis of the squalene hypothesis. Thus, in view of these results, when compared with those obtained from cholesterol[4-6] and from the tetracyclic triterpene, eburicoic acid,[5] it may be concluded that the biosynthesis of ergosterol follows the squalene pathway.[16]

To isolate C-11 and C-12 of ring C,C^{14}-ergosterol, obtained from *Saccharomyces cerevisiae* grown in the presence of 1-C^{14}-acetate,[17] was converted in a 7-step procedure to 3β-acetoxyergostan-11-one (IX) by a combination of published procedures.[18-21] This material then served as a common starting point for the degradations which yielded the individual carbon atoms.

For the degradation leading to C-11, IX was converted to 3β,11-diacetoxyergost-9(11)-ene (X)[22] which, in turn, was ozonized and saponified to yield 3β-hydroxy-9-keto-9,11-secoergostan-11-oic acid (XI). The acid XI was decarboxylated by allowing it to react with sodium azide in sulfuric acid and the evolved carbon dioxide (C-11 of ergosterol) collected.

For the degradation leading to C-12, IX was first reduced with sodium borohydride[22] and the resulting 11β-ol dehydrated with boron trifluoride to yield 3β-acetoxyergost-9(11)-ene (XII). The olefin XII then was oxidized with *t*-butyl chromate and the resulting 3β-acetoxyergost-9(11)-en-12-one (XIII) was hydrogenated over Pd–$CaCO_3$ catalyst to form the saturated 12-ketone XIV. The ketone was cleaved by allowing it to react with perbenzoic acid[23] and the ring C lactone which was formed was saponified to yield 3β,13-dihydroxy-12,13-secoergostan-12-oic acid (XV). The acid was decarboxylated by allowing it to react with sodium azide in sulfuric acid and the evolved carbon dioxide (C-12 of ergosterol) collected. As shown in Table I, both C-11 and C-12 of ring C which should be derived from the carboxyl of acetate were labeled and furthermore as demanded by the concept of the utilization of intact squalene, they were equally labeled.

Thus, the results of this study clearly show that the biosynthesis of the yeast sterol, ergosterol, follows the squalene hypothesis. In addition, the results obtained from the ring C degradation strengthen the concept that squalene is used as an

(15) W. G. Dauben, H. G. Wight and G. A. Boswell, *J. Org. Chem.*, **23**, 1787 (1958).

(16) In the previous work on side-chain degradation, an error in the specific activity determination of the starting ergosterol can readily account for the results obtained since all further calculations are based upon this value. Also, allowing for the fact that C-28 does not arise from acetate, recalculation of the doubly-labeled acetate experiment shows that 27 carbons are derived from acetate.

(17) We are indebted to Professor D. J. Hanahan, Department of Biochemistry, University of Washington, for kindly supplying us with the labeled sterol.

(18) W. V. Ruyle, E. M. Chamberlin, J. M. Chemerda, G. E. Sita, L. M. Aliminosa and R. L. Erickson, THIS JOURNAL, **74**, 5929 (1952).

(19) R. C. Anderson, R. Stevenson and F. S. Spring, *J. Chem. Soc.*, 2901 (1952).

(20) E. M. Chamberlin, W. V. Ruyle, A. E. Erickson, J. M. Chemerda, L. M. Aliminosa, R. L. Erickson, G. E. Sita and M. Tishler, THIS JOURNAL, **75**, 3477 (1953).

(21) P. Bladon, H. B. Henbest, E. R. H. Jones, B. J. Lovell, G. F. Woods, G. W. Wood, J. Elks, R. M. Evans, D. E. Hathaway, J. F. Oughton and G. H. Thomas, *J. Chem. Soc.*, 2921 (1953).

(22) A. Crawshaw, H. B. Henbest and E. R. H. Jones, *ibid.*, 731 (1954).

(23) E. S. Rothman, M. E. Wall and C. R. Eddy, THIS JOURNAL, **76**, 527 (1954).

intact unit when serving as a direct precursor of sterols and triterpenes.

Experimental[24]

Progesterone (V).—A mixture of 2.37 g. of unlabeled ergosterol and 0.130 g. of C^{14}-ergosterol was degraded to progesterone following the published procedure.[13] The yield of progesterone was 0.56 g. (27.9%), m.p. 124–127°, $[\alpha]^{25}D$ + 190° (Chf.) (lit.[13] m.p. 128–129°, $[\alpha]D$ +174° (Diox.).

5,20-Diketo-3,5-seco-A-norpregnan-3-oic Acid (VI).—A mixture of 0.220 g. of the above labeled progesterone and 1.80 g. of unlabeled material was recrystallized from methanol, yield 1.50 g. (75%). This diluted progesterone (1.00 g.) was dissolved in 18 ml. of glacial acetic acid and 18 ml. of ethyl acetate and then 1 ml. of freshly-boiled distilled water added. The resulting solution was cooled in an ice-salt bath and allowed to react with 8 mmoles of ozone (0.5 mmole per minute). The ozonolysis mixture was allowed to come to room temperature and remain at that temperature for 12 hr. during which time a white crystalline material formed. The mixture then was diluted with 50 ml. of benzene and the solvents distilled into an ice-cooled receiver. Two additional 25-ml. portions of benzene were added and distilled in the same manner. The distillate was treated with 3.0 g. of mercuric acetate and the evolved carbon dioxide swept into sodium hydroxide solution with a stream of nitrogen. The carbon dioxide then was precipitated with a mixture of ammonium chloride and barium chloride, filtered, dried at 100° (5 mm.) for 24 hr., yield 140 mg. (23%).

The residue from the above distillation of solvents was triturated with ether and the white precipitate filtered, yield of diketo acid VI, 653 mg. (62%), m.p. 175–176° (lit.[25] m.p. 173–175°).

3,5-Seco-A-norpregnan-3-oic Acid (VII).—To a solution of 3.0 g. of potassium hydroxide in 25 ml. of redistilled diethylene glycol, there was added 2.5 ml. of 85% hydrazine hydrate and 0.65 g. of diketo acid VI in 3 ml. of hot ethanol. The solution was refluxed for 1 hr. (bath temp. 135°), the reflux condenser removed and solvent allowed to distil until the internal temperature of the reaction reached 200°. The condenser then was replaced and the solution allowed to reflux for 4 hours. The clear solution was poured into 200 ml. of water and acidified with concentrated hydrochloric acid. The white precipitate was filtered and recrystallized from aqueous acetone, yield 0.46 g. (76%), m.p. 149–150°, $[\alpha]^{20}D$ +11.7° (*c* 0.84, Chf.) (lit.[25] m.p. 141–145°, $[\alpha]$ +8°).

3,4-Bisnor-2,5-seco-2-aminopregnane (VIII).—A solution of 0.40 g. of desketo acid VII in 7 ml. of dry chloroform was placed in a 50-ml. flask equipped with a magnetic stirrer and attached to a carbon dioxide collection train. Then, 2 ml. of 100% sulfuric acid was added with stirring and while sweeping the mixture with nitrogen, 0.127 g. of purified sodium azide was added through an addition tube. The entire operation was conducted at 0° and then the mixture was allowed to come to room temperature and stirred overnight. Subsequently, the reaction mixture was heated to 50° for 1 hr. and then the collected carbon dioxide precipitated as barium carbonate in the usual manner, yield 261 mg. (97%).

The acidic reaction mixture was diluted with ether and then sodium hydroxide solution added until the mixture was just alkaline. The organic layer was separated, washed with sodium hydroxide, water, dried and the solvent removed. The residue was dissolved in 10 ml. of acetic anhydride and allowed to stand at room temperature for 10 hr. The solution was evaporated under reduced pressure and the acetyl derivative recrystallized from hexane, m.p. 122–123°.

Anal. Calcd. for $C_{19}H_{36}ON$: N, 4.74. Found: N, 4.68.

3β-Acetoxy-7,9(11), 22-ergostatriene.—A solution of 8.14 g. of C^{14}-ergosteryl acetate in 50 ml. of benzene was hydrogenated over Raney nickel[18] and the 7,22-diene recrystallized from ethyl acetate, yield 7.09 g. (87%), m.p. 178–180°, $[\alpha]^{22}D$ −18° (*c* 1.2, chf.) (lit.[18] m.p. 184–187°, $[\alpha]$ −19.2°). To the mother liquor then was added 500 mg. of unlabeled material and by recrystallization an additional 970 mg. of product, m.p. 169–173°, $[\alpha]^{25}D$ −17° (*c* 1.07, Chf.) was obtained.

The combined 7,22-diene (8.07 g.) was converted to the 22,23-dibromo-7,9(11)-diene by reaction with bromine followed by treatment with sodium iodide,[19] yield 3.24 g. (30%), m.p. 220–224° dec. The dibromide (3.19 g.) was diluted with 0.90 g. of unlabeled material and then debrominated with zinc to yield 2.85 g. (94%) of 3β-acetoxy-7,9(11),22-ergostatriene, m.p. 176–178°, $[\alpha]^{25}D$ +32° (*c* 1.23, Chf.), λ_{max}^{EtOH} 242 mμ (ε 17,400), (lit.[19] m.p. 178–180°, $[\alpha]$ +32°, λ_{max}^{EtOH} 242 mμ (ε 19,000)).

3β-Acetoxyergostan-11-one (IX).—A solution of 2.845 g. of C^{14}-3β-acetoxy-7,9(11),22-ergostatriene in 30 ml. of benzene was allowed to react with a benzene solution of perbenzoic acid in the usual manner.[20] After processing there was obtained 1.86 g. (63%) of the 9α, 11α-oxide derivative, m.p. 204–206°, $[\alpha]^{25}D$ −35° (*c* 1.27, Chf.) (lit[20] m.p. 211–212°, $[\alpha]$ −39°).

The oxide (1.85 g.) was allowed to react with boron trifluoride etherate and the 3β-acetoxy-9β-ergosta-7,22-dien-11-one isolated in the described manner,[21] yield 1.61 g. (87%), m.p. 153.0–154.5°, $[\alpha]^{25}D$ −171° (*c* 1.11, Chf.). This material was hydrogenated [21] in ethyl acetate–acetic acid over platinum oxide and then recrystallized from methanol–chloroform to yield 1.51 g. (93%) of 3β-acetoxy-9β-ergostan-11-one, m.p. 149–151°, $[\alpha]^{25}D$ +44° (*c* 1.05, Chf.). The 9β-isomer (1.40 g.) was isomerized to the 9α-isomer by allowing the material to stand in contact with potassium hydroxide solution,[21] yield 1.27 g. (91%), m.p. 132–133°, $[\alpha]^{25}D$ +32° (*c* 1.00, Chf.) (lit.[21] m.p. 135–136°, $[\alpha]$ +33°).

3β-Acetoxy-9-keto-9,11-secoergostan-11-oic Acid.—A solution containing 1.87 g. (0.227 g. of labeled plus 1.643 g. of unlabeled) of IX, 0.42 g. of *p*-toluenesulfonic acid monohydrate and 155 ml. of acetic anhydride was heated for a period of 29 hr. while the volatile components were allowed to distil slowly through a 3″ Vigreux column. At the end of this period the remaining solvent was removed under reduced pressure, the dark oily residue dissolved in 300 ml. of a 1:1 mixture of pentane–benzene, washed with 5% sodium bicarbonate solution and then dried. The solvent was evaporated and the dark residue was filtered through a 2.5 × 11 cm. column of neutral alumina (Woelm). The column was further eluted with a mixture of 9:1 benzene–ether and concentration of the combined eluates gave 1.70 g. of crude enol acetate containing some starting ketone.[22] This material was dissolved in 40 ml. of methylene chloride, the solution cooled to −70° and then treated with a solution of 2.35 mmoles of ozone in 70 ml. of methylene chloride which was at the same temperature. The reaction mixture was maintained at −70° for 10 minutes at which time the blue color still remained, then allowed to warm to room temperature and the solvent removed under reduced pressure at 20°. The residue was dissolved in 25 ml. of acetic acid, a small volume of the acid distilled under reduced pressure to eliminate the residual amount of methylene chloride, then 0.5 ml. of water and 25 ml. of benzene were added. The mixture was treated with two 5-g. portions of zinc dust, with stirring. After the mixture had been stirred for 45 minutes, it was allowed to stand overnight, benzene added to the reaction, the zinc filtered and the acetic acid washed out with water. The benzene solution was concentrated and the residue chromatographed on 47 g. of Florex-XXS. With benzene, the remaining starting ketone was eluted and the keto acid was removed with benzene and benzene–ether. This latter compound was recrystallized from acetonitrile, yield 0.535 g. (27%), m.p. 146–147°, $[\alpha]^{25}D$ −57° (*c* 0.96, Chf.). The analytical sample has m.p. 148.0–148.2°, $[\alpha]^{25}D$ −55° (*c* 1.05, Chf.).

Anal. Calcd. for $C_{30}H_{50}O_5$: C, 73.43; H, 10.27; neut. equiv., 491. Found: C, 73.33; H, 10.13; neut. equiv., 484.

3β-Hydroxy-9-keto-9,11-secoergostan-11-oic Acid (XI).—The corresponding 3β-acetoxy derivative (0.535 g.) was refluxed for 1 hr. with a solution of 0.270 g. of potassium hydroxide in 20 ml. of methanol. After acidification and extraction with methylene chloride, the compound was recrystallized twice from acetonitrile, yield 0.445 g. (91%), m.p. 172.5–173.5°. $[\alpha]^{25}D$ −57° (*c* 1.22, Chf.).

Anal. Calcd. for $C_{28}H_{48}O_4$: C, 74.95; H, 10.78; neut. equiv., 449. Found: C, 74.82; H, 10.75; neut. equiv., 449.

The acid was decarboxylated by reaction with sodium azide and sulfuric acid in the manner described above. From

(24) All analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California. Radioactivity determinations were performed with thin mica window Geiger–Müller tube or with a vibrating reed electrometer. All m.p. are corrected.

(25) T. Reichstein and H. G. Fuchs, *Helv. Chim. Acta*, **23**, 676 (1940).

399 mg. of acid, there was obtained 179 mg. (102%) of barium carbonate.

3β-Acetoxy-11-β-hydroxyergostane.—A solution of 2.82 g. of C^{14}-3β-acetoxy-ergostan-11-one in 50 ml. of purified dioxane was allowed to react with a solution of 0.5 g. of sodium borohydride in aqueous methanol in the usual manner.[22] The crude product was acetylated with acetic anhydride and then recrystallized from methanol–ethyl acetate, yield 2.40 g. (85%), m.p. 129–129.5°; $[\alpha]^{25}D$ +22° (*c* 1.23, Chf.) (lit.[22] m.p. 134–135°, [α] +25).

3β-Acetoxyergost-9(11)-ene (XII).—A solution of 2.40 g. of C^{14}-3β-acetoxy-11β-hydroxyergostane in 15 ml. of glacial acetic acid and 25 ml. of purified dioxane was treated with 7 ml. of freshly distilled boron trifluoride etherate and the mixture allowed to stand for 24 hr. at room temperature.[22] After neutralization with 5% sodium bicarbonate solution, the product was extracted with pentane and recrystallized twice from methanol–chloroform, yield 1.90 g. (83%), m.p. 125–126°, $[\alpha]^{25}D$ +17° (*c* 1.25, Chf.) (lit.[22] m.p. 128–129°, [α] +17°).

When the oily residues from the preparation of 3β-acetoxy-11β-hydroxyergostane were treated similarly with boron trifluoride etherate and the reaction product chromatographed and recrystallized, an additional 0.38 g. of XII, m.p. 124–125°, was obtained.

3β-Acetoxyergost-9(11)-en-12-one (XIII).—To a round-bottomed 100-ml. flask fitted with a stirrer, dropping funnel and reflux condenser was added a solution of 2.28 g. of C^{14}-3β-acetoxyergost-9(11)-ene in 20 ml. of carbon tetrachloride. The flask was heated to 85° and a solution of 32 mmoles of *t*-butyl chromate (in carbon tetrachloride)[26] in 10 ml. of glacial acetic acid and 5 ml. of acetic anhydride was added slowly over a period of 30 minutes. During the reaction, the flask was swept with a stream of dry nitrogen. After 6 hr. at the elevated temperature, the flask was cooled in an ice-bath. After diluting the reaction mixture with 5 g. of ice, 6 g. of oxalic acid dihydrate was added slowly, with stirring. The organic layer was separated and the aqueous layer extracted twice with 25-ml. portions of carbon tetrachloride. The combined organic layers were washed with water, 5% hydrochloric acid, 5% sodium bicarbonate and water. The solvent was evaporated, the residual oil dissolved in 1:1 pentane–ether (absolute) and chromatographed on neutral Woelm alumina. From the pentane–ether (400 ml.) and ether (200 ml.) eluates there was obtained 1.95 g. of crude unsaturated ketone which was recrystallized from 30 ml. of pentane. A first crop of 1.20 g. (51%) and second crop of 0.28 g. (12%) were obtained, m.p. 130–131°. The analytical sample had m.p. 133.5–134.2°, $[\alpha]^{25}D$ +45° (*c* 1.61, Chf.), λ_{max}^{EtOH} 238 mμ (ε 10,900).

Anal. Calcd. for $C_{30}H_{48}O_3$: C, 78.90; H, 10.59. Found: C, 79.12; H, 10.62.

Saponification with potassium hydroxide in methanol of an unlabeled sample of acetate yielded 3β-hydroxyergost-9(11)-en-12-one. The compound after recrystallization from hexane–benzene has a m.p. 145.5–146.8°, $[\alpha]^{25}D$ +48° (*c* 1.49, Chf.). λ_{max}^{EtOH} 238 mμ (ε 10,800).

Anal. Calcd. for $C_{28}H_{46}O_2$: C, 81.10; H, 11.18. Found: C, 80.84; H, 11.12.

3β-Acetoxyergostan-12-one (XIV).—A solution of 1.48 g. of C^{14}-3β-acetoxyergost-9(11)-en-12-one in 50 ml. of ethyl acetate was hydrogenated at atmospheric pressure over 230 mg. of 10% Pd–$CaCO_3$. One mole equivalent of hydrogen was absorbed in 6.5 hr. The product was recrystallized from methanol–chloroform to yield 1.25 g. (84%) of product, m.p. 174–175°, $[\alpha]^{25}D$ +53° (*c* 1.12, Chf.).

Anal. Calcd. for $C_{30}H_{50}O_3$: C, 78.55; H, 10.99. Found: C, 78.31; H, 10.83.

3β,13-Dihydroxy-12,13-secoergostan-12-oic Acid (XV).—A solution of 1.25 g. of C^{11}-3β-acetoxyergostan-12-one and 5.5 mmoles of perbenzoic acid in 15 ml. of *moist* chloroform and 15 ml. of glacial acetic acid containing 0.2 ml. of 10% sulfuric acid was allowed to stand in the dark at room temperature for 8 days. The reaction mixture was diluted with ether and the organic layer washed with water, aqueous sodium sulfite, 3% sodium hydroxide solution and water. The solvents were removed under reduced pressure and the residue recrystallized twice from methanol to afford 284 mg. (24%) of 3β,13-dihydroxy-12, 13-secoergostan-12-oic acid-12,13-lactone, m.p. 186–190°, $[\alpha]^{25}D$ −7° (*c* 1.06, Chf.). The material remaining in the mother liquor was chromatographed on 120 g. of Florex XXS to yield an additional 217 mg. (18%) of product. The analytical sample had m.p. 195.5–196.5°, $[\alpha]^{25}D$ −8° (*c* 1.19, Chf.).

Anal. Calcd. for $C_{28}H_{48}O_3$: C, 77.71; H, 11.18. Found: C, 77.82; H, 11.43.

The above crude hydroxyl-acetone was combined and refluxed for 12 hr. with 1.2 g. of potassium hydroxide in 12 ml. of methanol and 3 ml. of water. Most of the methanol was distilled, 10 ml. of water added and the clear solution acidified at 0° with 20% aqueous acetic acid. The crude acid was recrystallized from methanol–acetonitrile to yield 338 mg. (65%) of product, m.p. 182–183° dec. The analytical sample has a m.p. 188.5–189.3° and $[\alpha]^{25}D$ 0° (*c* 1.09, MeOH).

Anal. Calcd. for $C_{28}H_{50}O_4$: C, 74.61; H, 11.18; neut. equiv., 451. Found: C, 74.36; H, 11.18; neut. equiv., 431.

The acid was decarboxylated by reaction with sodium azide and sulfuric acid in the manner described above. From 300 mg. of acid, there was obtained 78 mg. (60%) of barium carbonate.

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(26) K. Heusler and A. Wettstein, *Helv. Chim. Acta*, **35**, 284 (1952).