(2) Antagonism between Ba^{2+} and strongly basic antispasmodics (pKa>8.5) of II-group was competitive and that between Ba^{+2} and papaverine of I-group, which would exert through some physicochemical property of non-ionized molecules, was non-competitive on all the smooth muscle preparations used.

(3) Since the antibarium action is qualitatively different between I-group and II-group antispasmodics, the comparison of the potency ratio to papaverine as standard must be made under careful consideration.

(4) ACh and atropine-like antispasmodics were proved to have higher potency on intact ileum and longitudinal muscle strip than on circular muscle strip with and without ganglion cells.

(5) Atropine inhibited the nicotine-contraction of guinea pig ileum non-competitively in 1000 times or higher concentration than that at which atropine competitively antagonizes the contraction by ACh.

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32. Makoto Shirasaka and Masako Tsuruta : Microbiological Transformation of Steroid. V.[1] Hydroxylation of Steroid by *Sclerotium hydrophilum*.

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Among the hydroxylation of steroids by fungi, 11α-hydroxylation is comparatively common but this is often accompanied by 6β-hydroxylation in majority of cases. *Rhizopus arrhizus* used by Peterson and others[2] is the representative of such fungi.

During examination of numerous fungi, *Sclerotium hydrophilum* was found to carry out 11α- and 6β-hydroxylation of Reichstein's compound S at the same time. Application of this fungus to various other steroids showed the formation of chiefly 6β,11α-dihydroxy compound from progesterone and deoxycorticosterone, about equal amounts of 6β- and 11α-hydroxy compounds from 17α-hydroxyprogesterone, as in the case of the compound S, and 6β- and 15β-hydroxy-11-oxo compounds from corticosterone. Consequently, this fungus was found to have a kind of substrate specificity.

Fermentation of *Sclerotium hydrophilum* using potato decoction as a medium and by shake culture, as will be described later, with progesterone as the substrate and paper chromatographic examination of the concentrated ethyl acetate extract showed the presence of unreacted progesterone and a spot with much greater polarity than that. The concentrate was dissolved in benzene with warming and the crude crystals that separated on cooling were recrystallized from methanol to granular crystals (I) of m.p. 236~241°. Its analytical values indicated it to be dihydroxyprogesterone and the constants of (I) and its diacetate, obtained by the usual acetylation with acetic anhydride and pyridine, and their infrared spectra, were in good agreement with those of 6β,11α-dihydroxyprogesterone[1] and its 6,11-diacetate.[1]

The same fermentation of this fungus with 17α-hydroxyprogesterone as the substrate and paper chromatographic examination of the concentrated extract showed the presence of some unreacted 17α-hydroxyprogesterone and two spots with greater polarity than that.

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1) Part IV : This Bulletin, **9**, 159 (1961).

2) D. H. Peterson, *et al.* : J. Am. Chem. Soc., **74**, 5933 (1952); **75**, 408, 412, 416 (1953).

These substances were separated by Florisil-column chromatography and two crystalline substances were obtained, one of m.p. 238~240°(II) and the other of m.p. 214~224°(III), besides a minute amount of the unreacted substrate steroid. Analytical values showed that both are dihydroxyprogesterone, and both formed a monoacetate on acetylation with acetic anhydride and pyridine, indicating that the newly introduced hydroxyl is primary or secondary in both cases. The constants and infrared spectra of (II) and its acetate were identical with those of 6β,17α-dihydroxyprogesterone[3] and its 6-acetate, while those of (III) and its acetate were identical with the corresponding one of 11α,17α-dihydroxyprogesterone[4] and its acetate. Consequently, (II) was established as 6β,17α-dihydroxy progesterone and (III) as 11α,17α-dihydroxyprogesterone.

The same fermentation of this fungus with deoxycorticosterone as the substrate resulted in marked inhibition of fungal growth and oxidation reaction was found to be very weak. It is therefore considered that deoxycorticosterone has some kind of toxicity against this fungus.

The use of 21-acetate in place of free deoxycorticosterone showed that there was no inhibitory effect on fungal growth and paper chromatographic examination of concentrated extract indicated that oxidation had progressed smoothly. The paper chromatogram showed the presence of one spot with extremely great polarity. The concentrate was mixed with ethylene dichloride, the insoluble matter was collected by filtration, and recrystallized several times from methanol. Final recrystallization from acetone gave crystals (IV) of m.p. 220~226° and its analytical values indicated that two hydroxyls had been introduced into deoxycorticosterone. Acetylation of (IV) with acetic anhydride and pyridine afforded a triacetate, indicating that the newly introduced two hydroxyls are both primary or secondary.

Mild reduction of (IV) with zinc dust resulted in liberation of one hydroxyl and a dihydroxy-steroid was obtained, whose constants and infrared spectrum were identical with those of 11α,21-dihydroxypregn-4-ene-3,20-dione.[1] Therefore, one of the newly introduced hydroxyls must be in 11α-position. The hydroxyl liberated by the foregoing zinc reduction may be at 2- or 6-position.[5] Since the ultraviolet absorption maximum of (VI) is at 235.5 mμ, which is somewhat in a shorter wave-length region than that in ordinary Δ^4-3-oxo-steroids, this hydroxyl is considered to be in 6β-position. The difference in molecular rotation between (VI) and 11α,21-dihydroxypregn-4-ene-3,20-dione of ΔM_D −224 is similar to that of ΔM_D −252[3] between 6β-hydroxyprogesterone and progesterone, and of ΔM_D −250[3] between 6β,11α-dihydroxyprogesterone and 11α-hydroxyprogesterone, which all suggest that this hydroxyl is in 6β-position. Consequently, the structure of (VI) has been established as 6β,11α,21-trihydroxypregen-4-ene-3,20-dione, which is a new steroid.

Chromatography of the foregoing ethylene dichloride filtrate through a column of Florisil afforded a small amount of two kinds of crystals. The initially eluted fraction gave crystals of m.p. 190~198°(V) and the next one gave crystals of m.p. 156~160°(VI). Both gave analytical results indicating introduction of one hydroxyl into deoxycorticosterone. The constants and infrared spectrum of (V) agreed with those of 6β,21-dihydroxypregn-4-ene-3,20-dione[3] and no depression of m.p. occurred on admixture with an authentic sample. The constants and infrared spectrum of (VI) were identical with those of 11α,21-dihydroxypregn-4-ene-3,20-dione[1] and no depression of melting point was observed on admixture with an authentic sample.

Fermentation of this fungus with Reichstein's compound S and paper chromatographic examination of the concentrated extract indicated two spots of about equal quantity, with

3) Part III : This Bulletin, **9**, 152 (1961).
4) Part II : *Ibid.*, **9**, 59 (1961).
5) F. Sondheimer, *et al.* : J. Am. Chem. Soc., **75**, 4912 (1953).

greater polarity than compound S. Fractionation by Florisil-column chromatography afforded crystals (VII) of m.p. 231~238° from the initial eluate and crystals (VIII) of m.p. 205~210° from the following eluate. Both gave analytical results that indicated introduction of one hydroxyl into the compound S and both formed a diacetate on acetylation with acetic anhydride and pyridine. The constants of (VIII) were identical with those of 6β,17α,21-trihydroxypregn-4-ene-3,20-dione[3] and their admixture showed no depression of melting point. The constants of (VII) were identical with those of epihydrocortisone[1,4] and no depression of melting point occurred on admixture with authentic sample. The yield of (VII) and (VIII) was almost the same.

Finally, fermentation of this fungus was carried out with corticosterone as the substrate and paper chromatogaphic examination of the concentrated extract showed two spots with greater polarity than corticosterone. Fractionation by column chromatography first afforded some unreacted corticosterone and the following eluate afforded crystals (IX) of m.p. 195~200°. The final eluate gave crystals (X) of m.p. 195~200°. Analytical values of these two products indicated introduction of one hydroxyl into corticosterone and both formed a diacetate by usual acetylation with acetic anhydride and pyridine. Since the infrared spectrum of these two acetates did not show the absorption of a hydroxyl, 11β-hydroxyl in corticosterone was considered to have disappeared. The ultraviolet absorption maxima in (IX) and (X) were at 230.5 and 237.5 mμ, which are in shorter wave-length region than that of ordinary Δ^4-3-oxo-steroids, it was also assumed that the 11β-hydroxyl in corticosterone had been oxidized to 11-ketone group.[6]

Mild reduction of (IX) with zinc dust afforded a compound of m.p. 177~179°, formed by elimination of one hydroxyl. The constants of this substance agreed with those of 11-dehydrocorticosterone and their admixture failed to show any depression of melting point. It follows, therefore, that a ketone group is already present in 11-position. The ultraviolet absorption maximum of (IX) is at 230.5 mμ, which is in a shorter wave-length region than that (236 mμ) of 11-dehydrocorticosterone, and the newly introduced hydroxyl is thought to be in 6β-position. Consequently, the structure of (IX) was established as 6β,21-dihydroxypregn-4-ene-3,11,20-trione.

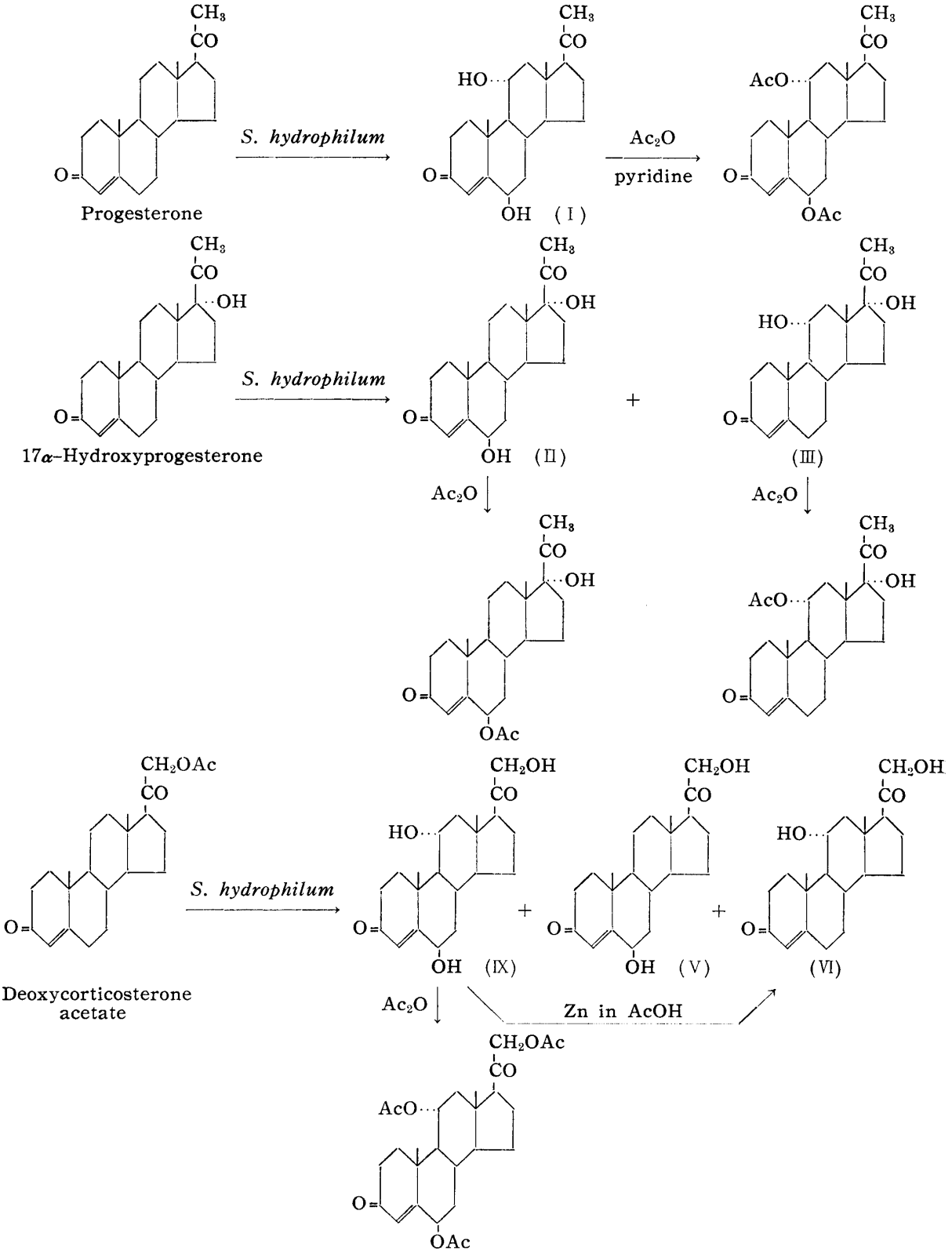
Acetylation of (X) with equivalent of acetic anhydride and pyridine, and oxidation of the reaction product directly with chromium trioxide, followed by hydrolysis of the oxidation product with methanolic solution of potassium hydrogencarbonate afforded a tetraketone compound. This substance has absorption at 1750 cm^{-1} in its infrared spectrum and the presence of a five-membered ketone may be assumed. Since this product was identical in its constants and infrared absorption data with 21-hydroxypregn-4-ene-3,11,15,20-tetrone, the five-membered cyclic ketone must be at 15-position, and the hydroxyl group newly introduced into (X) is at 15-position. The constants and infrared spectrum of the diacetate of (X) were identical with those of 15,21-diacetate of 15β,21-dihydroxypregn-4-ene-3,11,20-trione reported earlier[3] and the structure of (X) was therefore established as 15β,21-dihydroxypregn-4-ene-3,11,20-trione.

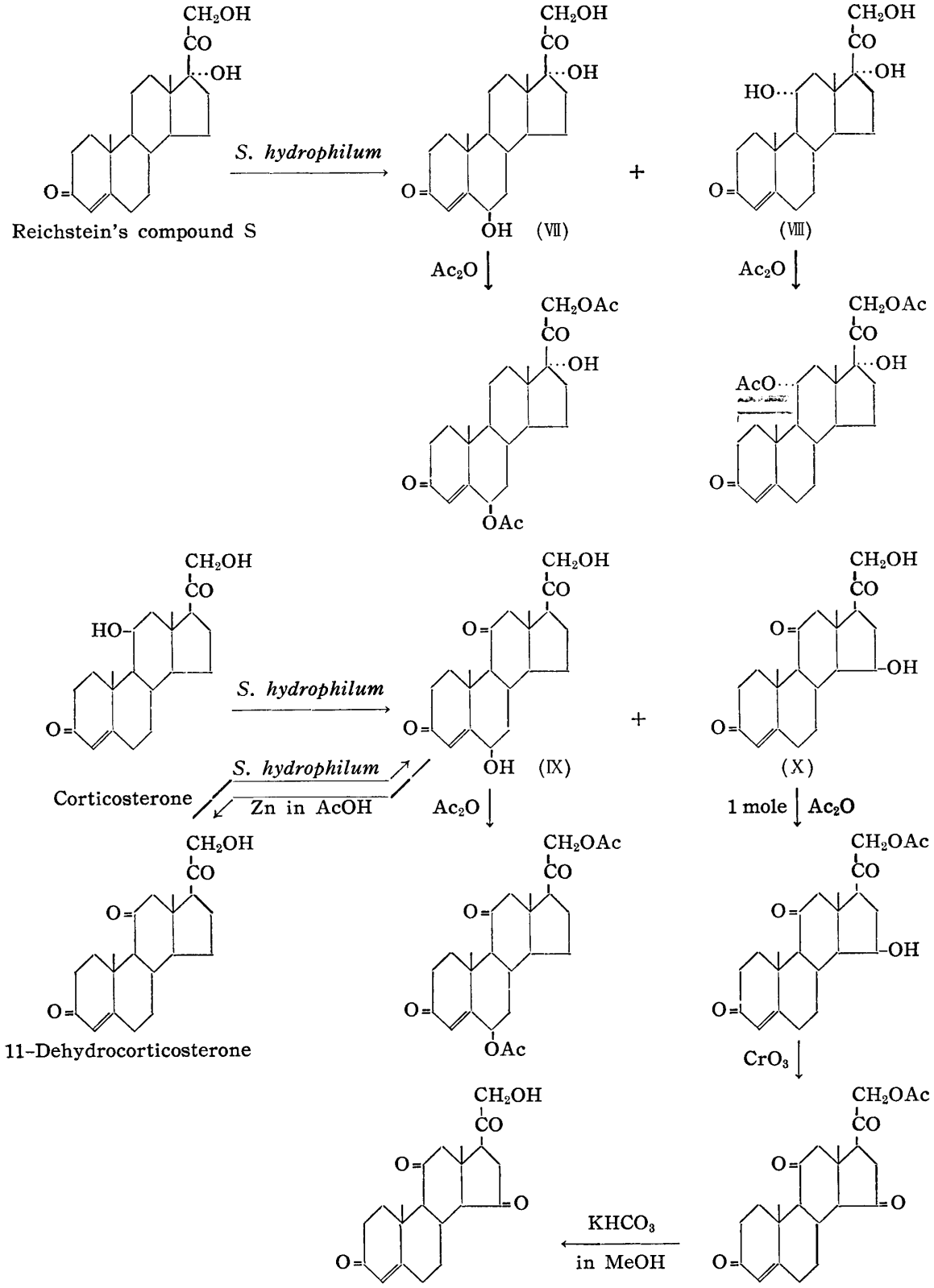
The same fermentation of this fungus with 11-dehydrocorticosterone, in place of corticosterone, as the substrate afforded (IX) and (X), but the same reaction with 11β,15β,21-trihydroxypregn-4-ene-3,20-dione failed to produce (X). Therefore, oxidation of corticosterone to (IX) and (X) is preceded by oxidation of 11β-hydroxyl in corticosterone and this is followed by 6β- or 15β-hydroxylation.

It was found from the result of foregoing experiments that *Sclerotium hydrophilum* effected 6β- and 11α-hydroxylation in about the same degree and similarly with all kinds of steroid. Moreover, the fact that 6β,11α-dihydroxy compound is obtained from pro-

6) F. Sondheimer, *et al.* : J. Am. Chem. Soc., **76**, 5020 (1954); L. Dorfman : Chem. Revs., **53**, 72 (1953).

gesterone and deoxycorticosterone, and 6β- and 11α-hydroxy compounds from other substrate steroids indicates that the hydroxyl in 21-position differs from that in 11β- or 17α-position and has no inhibitive effect on the dihydroxylation by this fungus. It has been found that this fungus effects 15β-hydroxylation of corticosterone, which was not observed in other substrate steroids, and this shows one of substrate specificity of this fungus.





This is probably due to the presence of 11β-hydroxyl in corticosterone. This fungus was also found to oxidize the 11β-hydroxyl group in corticosterone to a ketone but this oxidation was not effected when a hydroxyl had already been introduced into the 15β-position and the fact suggests that this enzyme also has some kind of substrate specificity.

Experimental

Fermentation and Extraction—Potato decoction containing 3% of glucose was poured into twenty 500-cc. shake flasks, 100 cc. to each flask, and sterilized. *Sclerotium hydrophilum* was inoculated in each flask and the flasks were shaken at 26° for 48 hr. To each flask, 2 cc. of 2.5% MeOH solution of the substrate steroid was added and the flasks were again shaken for 48~72 hr. After completion of fermentation, the culture liquid was filtered to separate fungal cells and the cells were extracted with Me_2CO and AcOEt. The combined extract and filtrate was extracted with two portions of AcOEt, the combined AcOEt solution was washed with 2% $NaHCO_3$ solution and water, dried over anhyd. Na_2SO_4, and evaporated in a reduced pressure.

Paper Chromatography—The procedure was the same as that described in Part I of this series.[7]

Hydroxylation of Progesterone—The fermentation of this fungus as described above, with 1 g. of progesterone as the substrate, afforded 1.6 g. of concentrated extract which was dissolved in benzene with application of heat and the crystals formed on cooling the solution were recrystallized several times from MeOH to 370 mg. of 6β,11α-dihydroxyprogesterone (I), m.p. 236~241°; $[\alpha]_D$ +100° (MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.94. Found: C, 72.15; H, 8.80. UV: λ_{max}^{MeOH} 236 mμ (ε 14,500). IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 1695 (20-CO), 1670, 1621 (Δ^4-3-CO). Further crop of 72 mg. of crystals (I) was obtained from the MeOH mother liquor.

6,11-Diacetate of (I): Usual acetylation of (I) with Ac_2O and pyridine afforded 6,11-diacetate of m.p. 152~155°; $[\alpha]_D$ +72.6° (MeOH). *Anal.* Calcd. for $C_{25}H_{34}O_6$: C, 69.74; H, 7.96. Found: C, 69.10; H, 8.12.

Hydroxylation of 17α-Hydroxyprogesterone—The same fermentation of this fungus with 1 g. of 17α-hydroxyprogesterone as the substrate afforded 1.8 g. of concentrated extract which was dissolved in 100 cc. of $C_2H_4Cl_2$ and passed through a column of 80 g. of Florisil. Fractional elution with various mixtures of $C_2H_4Cl_2$ and Me_2CO gave recovery of 210 mg. of unreacted substrate steroid from the initial eluate. The residue from the second fraction was recrystallized from Me_2CO and 187 mg. of 6β,17α-dihydroxyprogesterone (II) was further recrystallized to pure crystals of m.p. 238~240°; $[\alpha]_D$ +8° ($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.44; H, 8.81. UV: λ_{max}^{MeOH} 238.5 mμ (ε 12,200). IR ν_{max}^{KBr} cm^{-1}: 3370 (OH), 1705 (20-CO), 1664, 1625 (Δ^4-3-CO).

6-Monoacetate of (II): Usual acetylation of (II) with Ac_2O and pyridine gave a monoacetate of m.p. 188~190°; $[\alpha]_D$ +18° ($CHCl_3$). *Anal.* Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.43; H, 8.61. UV: λ_{max}^{MeOH} 236 mμ (ε 12,100). IR ν_{max}^{KBr} cm^{-1}: 3370 (OH), 1740 (acetyl CO), 1770 (20-CO), 1670, 1623 (Δ^4-3-CO).

The second eluate was treated similarly and 220 mg. of 11α,17α-dihydroxyprogesterone (III) was obtained as crude crystals. Further recrystallization gave pure crystals of m.p. 214~224°; $[\alpha]_D$ +80° ($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.74. Found: C, 72.20; H, 8.60. UV: λ_{max}^{MeOH} 243 mμ (ε 15,200). IR ν_{max}^{KBr} cm^{-1}: 3430 (OH), 1692 (20-CO), 1668, 1610 (Δ^4-3-CO).

11-Monoacetate of (III): Usual acetylation of (III) with Ac_2O and pyridine gave the 11-acetate of m.p. 203~209°, $[\alpha]_D$ +76° ($CHCl_3$).

Hydroxylation of Deoxycorticosterone Acetate—The fermentation of this fungus with 1 g. of deoxycorticosterone 21-acetate as the substrate afforded 1.6 g. of concentrated extract which was dissolved in 100 cc. of $C_2H_4Cl_2$ and the collected insoluble matter was recrystallized from MeOH to 207 mg. of crude crystals of 6β,11α,21-trihydroxypregn-4-ene-3,20-dione (IV). Repeated recrystallization gave pure crystals of m.p. 220~226°; $[\alpha]_D$ +105° (MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34. Found: C, 69.90; H, 7.98. UV: λ_{max}^{MeOH} 235.5 mμ (ε 15,500). IR ν_{max}^{KBr} cm^{-1}: 3370 (OH), 1678 (20-CO), 1665, 1615 (Δ^4-3-CO).

6,11,21-Triacetate of (IV): Usual acetylation of (IV) with Ac_2O and pyridine afforded the triacetate of m.p. 152~153°; $[\alpha]_D$ +107° (MeOH). *Anal.* Calcd. for $C_{27}H_{36}O_8$: C, 66.37; H, 7.40. Found: C, 66.50; H, 7.43. IR ν_{max}^{KBr} cm^{-1}: 1755 (acetyl CO), 1720 (20-CO), 1680, 1623 (Δ^4-3-CO).

Similar treatment of the second fraction afforded 80 mg. of crude crystals of 6β,21-dihydroxypregn-4-ene-3,20-dione (V) which was recrystallized to pure crystals of m.p. 190~198°; $[\alpha]_D$ +105° (MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.74. Found: C, 73.00; H, 8.82. UV: λ_{max}^{MeOH} 236 mμ (ε 13,600). IR ν_{max}^{KBr} cm^{-1}: 3350 (OH), 1702 (20-CO), 1670, 1615 (Δ^4-3-CO).

The last eluate afforded 94 mg. of crude crystals of 11α,21-dihydroxypregn-4-ene-3,20-dione (VI) which was recrystallized from Me_2CO to pure crystals of m.p. 156~160°; $[\alpha]_D$ +173° (MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.74. Found: C, 73.00; H, 8.62. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3470 (OH), 1710 (20-CO), 1668, 1615 (Δ^4-3-CO).

Reduction of (IV) with Zn and AcOH—A solution of 100 mg. of (VI) dissolved in 3 cc. of AcOH, added with 200 mg. of Zn dust and 0.2 cc. of water, was stirred for 30 min. at room temperature,

7) Part I: This Bulletin, **9**, 54 (1961).

Zn was removed from the reaction mixture, and the solution was evaporated in reduced pressure. The residue was diluted with 10 cc. of water and extracted with CH_2Cl_2. The extract was washed with $NaHCO_3$ solution and water, dried over anhyd. Na_2SO_4, and the solvent was evaporated in reduced pressure. Recrystallization of its residue from Me_2CO afforded 43 mg. of crystals, m.p. 156~160°; $[\alpha]_D$ +173°(MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.74. Found : C, 73.00; H, 8.62. IR $\nu_{max}^{CHCl_3}$ cm^{-1} : 3470 (OH), 1710 (20-CO), 1668, 1615 (Δ^4-3-CO).

The infrared spectrum of this substance was identical with that of 11α,21-dihydroxypregn-4-ene-3,20-dione and admixture of this substance with authentic sample of the latter showed no depression of m.p.

Hydroxylation of Reichstein's Compound S—The usual fermentation of this fungus with 1 g. of Reichstein's compound S as the substrate afforded 2 g. of the concentrated extract which was chromatographed through Florisil column as described above. The initial eluate gave 250 mg. of crude crystals (from Me_2CO) of 6β,17α,21-trihydroxypregn-4-ene-3,20-dione (Ⅶ) and further recrystallization gave crystals of m.p. 231~238°; $[\alpha]_D$ +62°($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34. Found : C, 69.70; H, 8.21. UV : λ_{max}^{MeOH} 238 mμ (ε 132,000). IR ν_{max}^{KBr} cm^{-1} : 3433 (OH), 1710 (20-CO), 1662, 1615 (Δ^4-3-CO).

6,21-Diacetate of (Ⅶ) : Usual acetylation of (Ⅶ) with Ac_2O and pyridine gave the diacetate of m.p. 190~192°; $[\alpha]_D$ +97°($CHCl_3$). *Anal.* Calcd. for $C_{25}H_{34}O_7$: C, 67.24; H, 7.65. Found : C, 67.61; H, 7.44.

The second eluate furnished 240 mg. of crude crystals (from Me_2CO) of epihydrocorticosterone (Ⅷ) which, after further recrystallization, melted at 205~210°; $[\alpha]_D$ +112°(MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34. Found : C, 69.10; H, 8.66. UV : λ_{max}^{MeOH} 242 mμ (ε 14,000). IR ν_{max}^{KBr} cm^{-1}: 3432 (OH), 1715 (20-CO), 1655, 1615 (Δ^4-3-CO).

11,21-Diacetate of (Ⅷ) : Usual acetylation of (Ⅷ) gave a diacetate, m.p. 203~209°; $[\alpha]_D$ +110°(MeOH). *Anal.* Calcd. for $C_{25}H_{34}O_7$: C, 67.24; H, 7.68. Found : C, 67.10; H, 7.90.

Hydroxylation of Corticosterone—Fermentation of this fungus with 1 g. of corticosterone as the substrate afforded 1.7 g. of the concentrated extract which was treated by Florisil-column chromatography as described above. The initial eluate furnished 70 mg. of unreacted corticosterone and the following eluate gave 173 mg. of crude crystals of 6β,21-dihydroxypregn-4-ene-3,11,20-trione (Ⅸ) which, after further recrystallization, melted at 195~200°; $[\alpha]_D$ +146.5°(MeOH). *Anal.* Calcd. for $C_{21}H_{28}O_5$: C, 69.97; H, 7.83. Found : C, 69.91; H, 7.31. UV : λ_{max}^{MeOH} 230.5 mμ (ε 14,300). IR ν_{max}^{KBr} cm^{-1}: 3340 (OH), 1702 (20-CO), 1670, 1615 (Δ^4-3-CO).

6,21-Diacetate of (Ⅸ) : The usual acetylation of (Ⅸ) with Ac_2O and pyridine afforded the 6,21-diacetate of m.p. 182~184°. *Anal.* Calcd. for $C_{25}H_{32}O_7$: C, 69.58; H, 8.34. Found : C, 69.10; H, 8.12. IR ν_{max}^{KBr} cm^{-1} : 1750 (acetyl CO), 1699 (20-CO), 1680, 1625 (Δ^4-3-CO).

The final fraction was evaporated and recrystallized from Me_2CO to 220 mg. of crude crystals of 15β,21-dihydroxypregn-4-ene-3,11,20-trione (Ⅹ). Recrystallization from Me_2CO gave pure crystals of m.p. 195~200°; $[\alpha]_D$ +190°(MeOH). *Anal.* Calcd. for $C_{21}H_{28}O_5$: C, 69.97; H, 7.83. Found : C, 69.70; H, 7.74. UV : λ_{max}^{MeOH} 237.5 mμ (ε 16,500). IR ν_{max}^{KBr} cm^{-1} : 3430 (OH), 1714 (11-CO), 1694 (20-CO), 1665, 1615 (Δ^4-3-CO).

15,21-Diacetate of (Ⅹ) : Usual acetylation of (Ⅹ) with Ac_2O and pyridine afforded 15,21-diacetate of m.p. 193~197°; $[\alpha]_D$ +125.5°(MeOH). *Anal.* Calcd. for $C_{25}H_{32}O_7$: C, 69.58; H, 8.34. Found : C, 69.78; H, 8.21. IR ν_{max}^{KBr} cm^{-1} : 1730 (acetyl CO), 1714 (20-CO), 1670, 1615 (Δ^4-3-CO).

Oxidation of (X) with CrO_3—A solution of 100 mg. of (Ⅹ) dissolved in 2 cc. of pyridine containing 33 mg. of Ac_2O was acetylated in a usual manner and the concentrated residue obtained from extraction of the reaction mixture with CH_2Cl_2 was dissolved in 2 cc. of AcOH containing 20 mg. of CrO_3. The mixture was allowed to stand for 5 hr. at room temperature to effect oxidation and the extract of this reaction mixture was concentrated. The residue was washed with a small quantity of cold Me_2CO and dissolved in hydr. MeOH containing 70 mg. of $KHCO_3$. After allowing this mixture to stand over night, it was extracted with CH_2Cl_2 and the residue obtained after evaporation of CH_2Cl_2 was recrystallized from Me_2CO to 21-hydroxypregn-4-ene-3,11,15,20-tetrone, m.p. 218~222°. *Anal.* Calcd. for $C_{21}H_{26}O_5$: C, 70.37; H, 7.31. Found : C, 70.10; H, 7.44. IR ν_{max}^{KBr} cm^{-1} : 3410 (OH), 1950 (15-CO), 1732 (11-CO), 1715 (20-CO), 1643, 1620 (Δ^4-3-CO).

These constants and infrared spectral data were identical with those of the reported 21-hydroxypregn-4-ene-3,11,15,20-tetrone.

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Summary

Application of *Sclerotium hydrophilum* to progesterone, 17α-hodroxyprogesterone, deoxycorticosterone, Reichstein's compound S, and corticosterone as the substrate steroids afforded 11α- and 6β-hydroxy compounds from all except corticosterone, and the latter formed 6β- and 15β-hydroxypregn-4-ene-3,11,20-trione.

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33. Makoto Shirasaka : Microbiological Transformation of Steroid. VI.[1] Hydroxylation of Steroid by *Stachylidium bicolor*.

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Hydroxylation of steroid by microörganisms is now known to occur in almost all the positions in the steroidal skeleton.[2] Among such microörganisms, those effecting 11β-hydroxylation are of importance and of practical value because they can directly manufacture steroidal hormones like hydrocortisone, and some fungi have been found to date that carry out 11β-hydroxylation, like *Cunninghamella* sp.[3] and *Curvularia* sp.[4]

During examination of oxidative ability of numerous fungi to steroids, it was found that *Stachylidium bicolor* effected 11β-hydroxylation of Reichstein's compound S and the fungus was applied to deoxycorticosterone and other steroids. It was thereby found that the fungus effected 14α-hydroxylation as well as 11β-hydroxylation, and while the fungus produced hydrocortisone almost solely from the compound S, it formed 14α-hydroxy compound from deoxycorticosterone and only a trace of 11β-hydroxy compound was formed. The fungus carried out 11β-hydroxylation of 14α,21-dihydroxypregn-4-ene-3,20-dione but did not effect 14α-hydroxylation of corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione). These results indicated that this fungus had an extremely marked substrate specificity, and these experiments are described herein.

The cultured cells of *Stachylidium bicolor* were applied to Reichstein's compound S as the substrate and the concentrated extract from the reaction mixture was examined by paper chromatography.[1] One main spot with greater polarity than the compound S and a very weak spot with smaller polarity than that were detected on the chromatogram. These spots were separated by Florisil-column chromatography and a large amount of hydrocortisone (I) was obtained as crystals. Another crop of crystals was obtained but the amount obtained was so small that the substance was not identified. Acetylation of (I) with acetic anhydride and pyridine gave a monoacetate and its oxidation with chromium trioxide afforded a triketone compound. The constants of the oxidation product were

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1) Part V : This Bulletin, **9**, 196 (1961).
2) E. Vischer, A. Wettstein : Advances in Enzymol., **20**, 237 (1959).
3) F. R. Hanson, *et al.* : J. Am. Chem. Soc., **75**, 5369 (1953).
4) G. M. Shull, *et al.* : *Ibid.*, **77**, 763 (1955).