The author expresses his deep gratitude to Prof. K. Tsuda and Prof. T. Asai of The Institute of Applied Microbiology, University of Tokyo, for their kind and unfailing guidance throughout the course of the present work. He is indebted to Miss M. Tsuruta of this Laboratory for her technical cooperation.

Summary

Cultured cells of *Stachylidium bicolor* was applied to Reichstein's compound S, deoxycorticosterone, 14α,21-dihydroxypregn-4-ene-3,20-dione, and corticosterone. The compound S produced hydrocortisone, deoxycorticosterone produced 11β- and 14α-hydroxylated compounds, and 14α,21-dihydroxypregn-4-ene-3,20-dione gave the 11β-hydroxy compound. Oxidation product was not obtained from corticosterone.

(Received June 14, 1960)

UDC 547.92.07:542.98:576.882.8

34. **Makoto Shirasaka and Masako Tsuruta**: Microbiological Transformation of Steroid. VIII.[1] 1-Dehydrogenation of Steroid by Fungi.

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1-Dehydrogenation of steroid is one of the frequent reactions among transformation of steroids by microörganisms. The recent discovery that the activity of adrenocortical hormone increases by the introduction of a double bond in 1-2 position of the steroidal structure and appearance of new hormones like prednisone and prednisolone have made this 1-dehydrogenation reaction quite an important and practicable reaction. Most of the microörganisms which are known to carry out this reaction are bacteria but a few fungi have been reported as effecting this reaction.[2,3] One of them is *Didimella lycopersia*[3] which produces 1-dehydro compound from hydrocortisone.

During examination of various fungi for 1-dehydrogenation of steroids, it was found that *Gliocladium roseum*, *Helminthosporium turcicum*, and *Ophiobolus heterostropus* effected 1-dehydrogenation of Reichstein's compound S. Application of these fungi to other steroids, deoxycorticosterone, corticosterone, and cortisone resulted in formation of their 1-dehydro compounds. However, none of the fungi were able to produce the 1-dehydro compound from hydrocortisone and it was found that these fungi had a marked substrate specificity in the 1-dehydrogenation reaction of steroids.

First, the fermentation of *Gliocladium roseum* was carried out, as will be described later, with deoxycorticosterone as the substrate and the concentrated extract of the culture liquid was examined by paper chromatography, using propylene glycol-toluene system as the developing solvent. The chromatogram contained one spot with somewhat greater polarity than deoxycorticosterone. Purification of the concentrated extract by Florisil-column chromatography afforeded some crystals (I) of m.p. 185~191°; $[\alpha]_D$ +110°(MeOH), whose analytical values showed that there was no increase in the oxygen content. The

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1) Part VII: This Bulletin, **9**, 238 (1961).
2) E. Visher, *et al.*: Helv. Chim. Acta, **38**, 835 (1955).
3) *Idem*: *Ibid.*, **38**, 1502 (1955).

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infrared spectrum of (I) showed absorptions at 1665, 1628, and 1605 cm^{-1} ($\Delta^{1,4}$-3-CO), from which (I) was considered to be the 1-dehydro compound of deoxycorticosterone. Acetylation of (I) with acetic anhydride and pyridine gave a monoacetate, and (I) and this acetate were identified by their physical constants with 1-dehydro-deoxycorticosterone, i.e. 21-hydroxypregna-1,4-diene-3,20-dione and its 21-acetate.[2,3]

The same culture of *Gl. roseum* with Reichstein's compound S as the substrate and paper chromatographic examination of the concentrated extract showed one spot with slightly greater polarity than the compound S. This extract was purified through column chromatography and crystals (II) of m.p. 232~236°; $[\alpha]_D$ +80° ($CHCl_3$), were obtained. The analytical values of (II) showed there was no increase in oxygen content and its infrared spectrum showed absorptions at 1670, 1615, and 1605 cm^{-1} ($\Delta^{1,4}$-3-CO), indicating that (II) is a 1-dehydro compound of the compound S. Acetylation of (II) with acetic anhydride and pyridine gave a monoacetate, and (II) and this acetate were identified with 17α,21-dihydroxypregna-1,4-diene-3,20-dione through their physical constants.[2,3]

The same fermentation of *Gl. roseum* with corticosterone as the substrate and paper

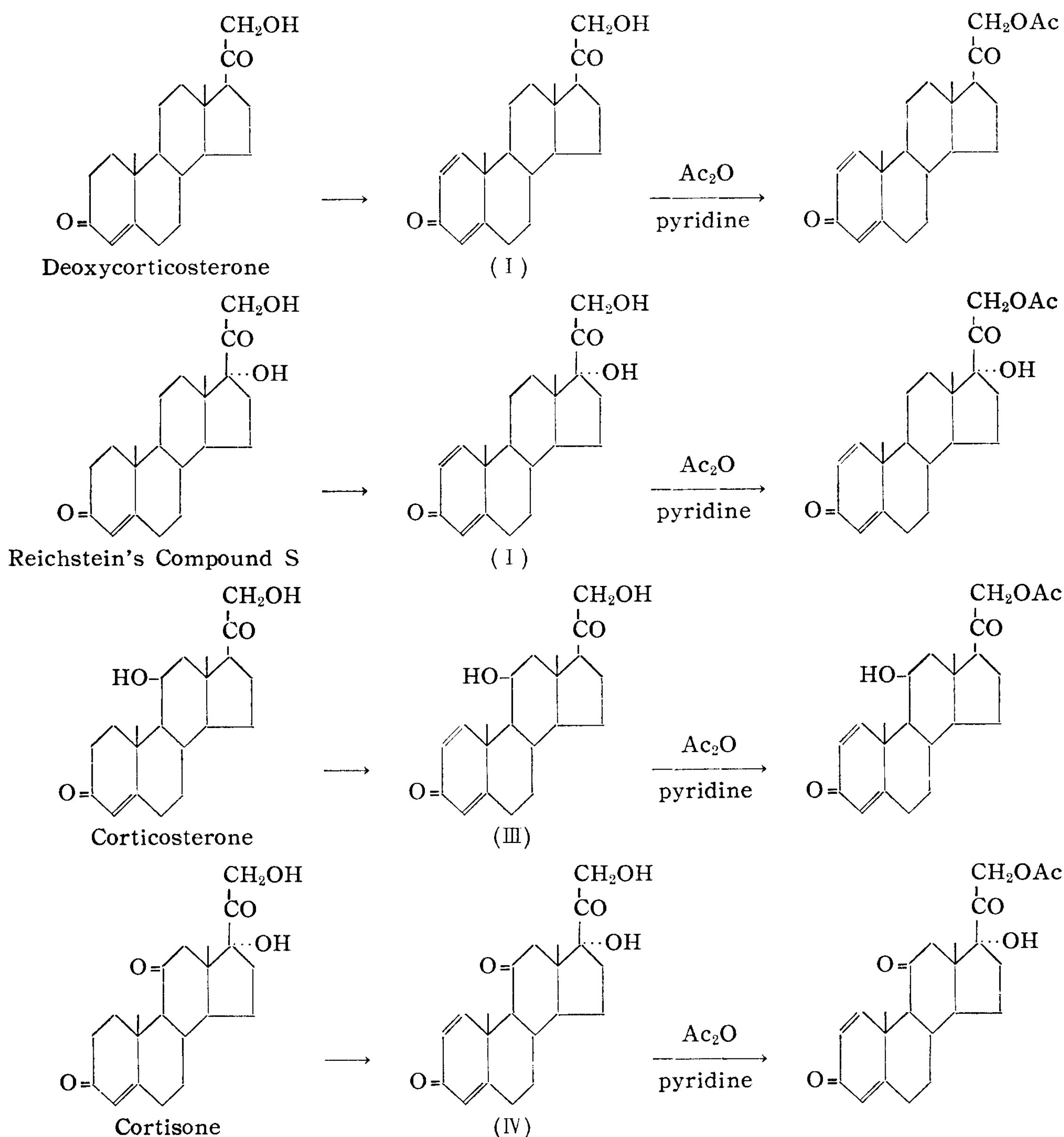

Chart 1.

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chromatographic examination of its concentrated extract showed a spot of corticosterone and another spot with somewhat greater polarity than that. These were purified through column chromatography and afforded unreacted corticosterone and some crystals (III) of m.p. 208～218°; $[\alpha]_D$ +140° (EtOH). The analytical values of (III) showed there had been no increase in oxygen content and infrared absorptions at 1660, 1645, and 1615 cm^{-1} ($\Delta^{1,4}$-3-CO) suggested that (III) is 11β,21-dihydroxypregna-1,4-diene-3,20-dione. Acetylation of (III) with acetic anhydride and pyridine gave a monoacetate, and (III) and this acetate were identified with 11β,21-dihydroxypregna-1,4-diene-3,20-dione and its 21-acetate through their physical constants.[2,3]

The same fermentation of *Gl. roseum* with cortisone as the substrate and paper chromatographic examination of its concentrated extract showed the presence of one spot with slightly greater polarity than cortisone. Direct recrystallization of this extract residue from acetone gave crystals (IV) of m.p. 230～234°; $[\alpha]_D$ +164° ($CHCl_3$). Its analytical values showed no increase in oxygen content and its infrared absorptions at 1670, 1625, and 1610 cm^{-1} suggested (IV) to be prednisone. Acetylation of (IV) with acetic anhydride and pyridine gave a monoacetate, and (IV) and this acetate agreed in physical constants with prednisone (17α,21-dihydroxypregna-1,4-diene-3,11,20-trione) and its 21-acetate.[2,3]

The same application of this fungus to hydrocoritsone and paper chromatographic examination of the concentrated extract of the fermentation liquor did not show the presence of any spot other than that of hydrocortisone, although there was a trace of a spot with somewhat greater polarity than that. It seems, therefore, that this fungus does not carry out 1-dehydrogenation of hydrocortisone, differing with other steroids described above.

Helminthosporium turcicum and *Ophiobolus heterostropus* were each cultured in the same manner as with *Gl. roseum*, using the same kinds of steroid as the substrate. Chromatographic examination of the resultant products showed that these three kinds of fungus carried out entirely the same reactions against each of these steroids, as indicated in Table I.

Table I. 1-Dehydrogenation of Various Steroids by Fungi

Substrate steroid	*Gliocladium roseum*	*Helminthosporium turcicum*	*Ophiobolus heterostropus*
Deoxycorticosterone	⧺	⧺	⧺
Reichstein's compound S	⧺	⧺	⧺
Corticosterone	+	+	+
Cortisone	⧺	⧺	⧺
Hydrocortisone	±	−	−

⧺ Almost no remains of unreacted substrate steroid
\+ Some unreacted substrate steroid remains
±, − Very slight or almost no reaction product found

These three kinds of fungus were found to carry out 1-dehydrogenation of all these steroids, except hydrocortisone, comparatively effectively. Since some unreacted substrate steroid remained in the case of 1-dehydrogenation of corticosterone, it seems this reaction is somewhat difficult in this steroid. These experimental results indicate that 1-dehydrogenation of steroids by these fungi is inhibited to a certain extent by the presence of a 11β-hydroxyl group in the substrate steroid and that these fungi have a kind of substrate specificity in this reaction.

Of these fungi, *Helminthosporium* and *Ophiobolus* spp. are Ascomycetes and imperfect fungi of the same system. Although they are clearly different in taxonomy and morphology, they show the same behavior in reaction with steroids and this seems to be a rather interesting point in the taxonomy of fungi.

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Experimental

Fermentation and Extraction—A medium consisting of 5% of glucose, 2% of peptone, and 0.3% of corn-steep liquor was placed in ten 500-cc. shake flasks, 100 cc. to each flask, the flasks were sterilized, and each was inoculated with one of the fungi. This was shake-cultured at 26° for 42～72 hr. To each of the flasks, 2 cc. of 2.5% MeOH solution of the substrate steroid was added and the flasks were again shake-cultured for 48～72 hr. After completion of the fermentation, fungal cells were separated from the liquid, cells were extracted with AcOEt, and the extract was combined with the filtrate. This combined filtrate was extracted twice with AcOEt, the combined extract was washed with 2% $NaHCO_3$ solution and water, dried over anhyd. Na_2SO_4, and the solvent was evaporated in reduced pressure. This concentrated extract was either crystallized directly from a suitable solvent or purified by column chromatography.

Paper Chromatography—The chromatography was carried out as described earlier,[4] by the descending method and using propylene glycol-toluene system as the developing solvent.

Column Chromatography—A solution of the foregoing concentrated extract dissolved in 50 cc. of $C_2H_4Cl_2$ was passed through a column of 40 g. of Florisil and the column was serially eluted with 40 cc. each of $C_2H_4Cl_2$ and the mixed solvent of $C_2H_4Cl_2$ and Me_2CO in various ratios (25:1, 20:1, 12:1, 10:1, 8:1, 5:1, 3:1). The combined fraction of the same substance was evaporated and the residue therefrom was recrystallized from Me_2CO, for purification and identification of each steroid.

1-Dehydrogenation of Deoxycorticosterone—*Gliocladium roseum* was fermented as described above, with deoxycorticosterone as the substrate and the extract was submitted to column chromatography. The fraction eluted by 10:1 mixture of $C_2H_4Cl_2$-Me_2CO afforded 315 mg. of crystals. Further recrystallization from Me_2CO afforded the crystals of (I), m.p. 185～191°; $[\alpha]_D$ +110°(MeOH). *Anal.* Calcd. for $C_{21}H_{28}O_3$: C, 80.73; H, 9.03. Found : C, 81.02; H, 8.92. UV : λ_{max}^{MeOH} 244 mμ (ε 14,300). IR ν_{max}^{KBr} cm^{-1} : 3450 (OH), 1690 (20-CO), 1665, 1628, 1605 ($\Delta^{1,4}$-3-CO).

21-Acetate of (I) : Usual acetylation of (I) with Ac_2O and pyridine gave the monoacetate of m.p. 204～206°; $[\alpha]_D$ +110°(MeOH). *Anal.* Calcd. for $C_{23}H_{30}O_4$: C, 74.56; H, 8.16. Found : C, 74.18; H, 8.22.

1-Dehydrogenation of Reichstein's Compound S—The same fermentation of *Gl. roseum* was carried out with the compound S as the substrate and 790 mg. of concentrated extract was obtained. This extract was submitted to the usual column chromatography and the fraction eluted with 8:1 mixture of $C_2H_4Cl_2$ and Me_2CO gave 306 mg. of crude crystals. Recrystallization from Me_2CO gave crystals of (II), m.p. 232～236°; $[\alpha]_D$ +80°($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{28}O_4$: C, 73.22; H, 8.19. Found : C, 73.61; H, 8.21. UV : λ_{max}^{MeOH} 244 mμ (ε 15,800). IR ν_{max}^{KBr} cm^{-1} : 3340 (OH), 1715 (20-CO), 1670, 1615, 1605 ($\Delta^{1,4}$-3-CO).

21-Acetate of (II) : Usual acetylation of (II) with Ac_2O and pyridine gave the monoacetate of m.p. 220～224°; $[\alpha]_D$ +82°($CHCl_3$). *Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82. Found : C, 71.61; H, 7.66.

1-Dehydrogenation of Corticosterone—The same fermentation of *Gl. roseum* with corticosterone as the substrate gave 690 mg. of the concentrated extract. Purification through the Florisil column and elution of the column with $C_2H_4Cl_2$-Me_2CO mixture gave 42 mg. of unreacted corticosterone from the fraction eluted with 12:1 mixture of these solvents. The fraction eluted with 8:1 and 5:1 mixtures of $C_2H_4Cl_2$ and Me_2CO was recrystallized from Me_2CO to 220 mg. of (III), m.p. 208～218°; $[\alpha]_D$ +140° (EtOH). *Anal.* Calcd. for $C_{21}H_{28}O_4$: C, 73.22; H, 8.19. Found : C, 73.41; H, 8.01. UV : λ_{max}^{MeOH} 244 mμ (ε 15,000). IR ν_{max}^{KBr} cm^{-1} : 3380 (OH), 1710 (20-CO), 1660, 1645, 1615 ($\Delta^{1,4}$-3-CO).

21-Acetate of (III) : Usual acetylation of (III) with Ac_2O and pyridine gave the monoacetate of m.p. 158～160°; $[\alpha]_D$ +145°(EtOH). *Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 8.82. Found : C, 71.33; H, 7.81.

1-Dehydrogenation of Cortisone—The same fermentation of *Gl. roseum* with cortisone as the substrate gave 820 mg. of the concentrated extract. This concentrated residue was dissolved in 50 cc. of Me_2CO with application of heat and 360 mg. of crude crystals of (IV) was obtained on cooling the solution. Several recrystallizations from Me_2CO gave pure crystals of (IV), m.p. 230～234°(decomp.); $[\alpha]_D$ +164°($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{26}O_5$: C, 70.37; H, 7.31. Found : C, 70.10; H, 7.43. UV: λ_{max}^{MeOH} 239 mμ (ε 15,000). IR ν_{max}^{KBr} cm^{-1} : 3330 (OH), 1713 (20-CO), 1670, 1625, 1610 ($\Delta^{1,4}$-3-CO). These infrared spectral data agreed with those of prednisone.

21-Acetate of (IV) : Usual acetylation of (IV) with Ac_2O and pyridine gave the monoacetate of m.p. 228～231°; $[\alpha]_D$ +183°($CHCl_3$). *Anal.* Calcd. for $C_{23}H_{28}O_6$: C, 69.98; H, 7.05. Found : C, 69.77; H, 7.20.

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

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The authors express their deep gratitude to Prof. K. Tsuda and Prof. T. Asai of The Institute for Applied Microbiology, University of Tokyo, for their unfailing guidance throughout the course of the present work and to Mr. M. Matsui, Director of this Laboratory, for kind encouragement.

Summary

1-Dehydro compounds were obtained from deoxycorticosterone, Reichstein's compound S, corticosterone, and cortisone by their fermentation with *Gliocladium roseum*, *Helminthosporium turcicum*, or *Ophiobolus heterostropus*.

(Received June 24, 1960)

UDC 547.456'118.5:577.155.2

35. Tyunosin Ukita and Masachika Irie : Organic Phosphates. X.[*2] Several 2,3-Cyclic Phosphates of D-Ribofuranoside and their Properties as Substrate for Ribonuclease.

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Recently, several reports were published on the mode of enzymatic hydrolysis reaction and the substrate specificity of pancreatic ribonuclease (RNase-I).

The RNase hydrolyzes ribonucleic acid specifically at C-O-P linkage at 5′-position of its internucleoside phosphate bonds which bind 3′-hydroxyl group of pyrimidine nucleoside to 5′-position of the neighboring nucleoside, and gives the final product of pyrimidine 3′-mononucleotides and oligonucleotides containing similar nucleotides as their terminal group. This reaction is known to include the corresponding pyrimidine 2′,3′-cyclic mononucleotides and the oligonucleotides with their terminal group of a similar cyclic nucleotide as intermediate compounds.[1,2]

Todd and Brown, in their research on the substrate specificity of this enzyme, found that both 2′,3′-cyclic and 3′-benzylphosphoryl derivatives of cytidine and uridine serve as its substrate to give the 3′-phosphates of these two nucleosides,[3] while the 2′-benzylphosphoryl derivatives of these pyrimidine nucleosides as well as 2′,3′-cyclic phosphate of adenosine and guanosine could not be attacked by this enzyme.

Durand, *et al.*[4] reported that apurinic acid obtained after removal of purine bases by acid treatment of deoxyribonucleic acid is slowly hydrolyzed by the RNase. Further, Chargaff[5] observed that the type-b specific substance separated from *Haemophilus influenzae*, the structure of which was proposed by him to be a polymer of 1,1′-ribosylriboside combined intermolecularly by phosphate linkages at their 3-5 and 3′-5′ hydroxyl groups, is capable of being hydrolyzed by relatively large amount of RNase.

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