Acknowledgment.—This research was supported in part by a research grant (A-4936) from the National Institutes of Health, Arthritis and Metabolic Diseases Division.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY 4, CALIF.]

The Configuration of B-Norsteroid Derivatives[1,2]

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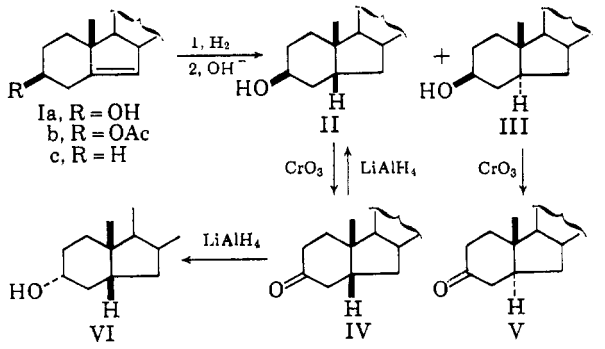
RECEIVED JANUARY 11, 1963

The stereochemical course of the hydrogenation of B-norcholesteryl acetate has been re-examined and it has been found that the major product possesses an A/B *cis* configuration, a result in direct contrast to that found with natural steroids. The epoxide derived from B-norcholesteryl acetate has been shown to possess the α-configuration. Studies of the reaction of the epoxide and its derivatives have permitted the determination of the configuration of "Butenandt diketone" (XIII) and the evaluation of the steric strain present in the B-norsteroid series. The configuration of the four isomeric diols derived from the above dione has been established and the certain reactions of the alcohols have been studied.

In the course of an earlier study of the reactions of B-norcholesterol,[4] it was found that when the modified sterol was hydrogenated in acetic acid mainly one isomer was formed (~75% yield). This major isomer was assigned an A/B *trans* configuration since such an isomer is formed in the similar reaction in the natural sterols.[5] Additional support for this stereochemical assignment was gained by examination of the products formed in the reduction by lithium aluminum hydride of the ketone derived from the hydrogenated alcohol. Subsequently, on the basis of optical rotatory dispersion measurements in this series, Djerassi, Marshall and Nakano[6] suggested that a *cis* arrangement of the A/B ring juncture was more likely. This problem of the nature of the ring juncture has now been re-examined, chemically, and it has been found that, indeed, the *cis* stereochemical assignment of the latter workers is correct.

First, the lithium aluminum hydride reduction of the saturated ketone IV was restudied and it was found that the earlier results were in error. Reduction of IV gave two isomers in a ratio of 3:1. The minor alcohol II was assigned the 3β-configuration since it was identical with the material prepared by hydrogenation of B-norcholesterol which has a 3β-hydroxyl group. In such a reduction of an unhindered ketone the equatorial isomer always is the major product[7] and, thus, the major product of the reduction, the 3α-hydroxyl isomer VI, must possess such a conformation, a conformational arrangement only possible with an A/B *cis* configuration.



To strengthen the optical rotatory argument, it was felt desirable to examine both the A/B *cis* and *trans* isomers in order to ensure no anomaly was present. By careful separation of the saponified hydrogenation products of B-norcholesteryl acetate (Ib) there was isolated in 70% yield the previously obtained major isomer II and, in addition, 15% of the minor isomer III. Oxidation of II and III yielded the corresponding 3-keto derivatives IV and V, the rotatory dispersion curves of which were practically identical with those of coprostanone and cholestanone, respectively.[8] This finding of similarity of optical rotatory dispersion curves in the normal stereoidal ketones and the related B-nor derivatives is most interesting since, as was pointed out by Djerassi,[6] rotatory dispersion would have been expected to be more sensitive to minor conformational alterations than catalytic hydrogenation. After completion of this work, similar stereochemical conclusions were arrived at by three other groups of workers.[9-11]

In view of this decisive influence of a five-membered B-ring on the steric course of hydrogenation, the direction of attack by a chemical reagent on the 5,6-double bond was investigated. It was found that when B-norcholesteryl acetate (Ib) was allowed to react with monoperphthalic acid there was formed in high yield a single epoxide (VIIb), whereas cholesteryl acetate under similar reaction conditions gives rise to a mixture of the α- and β-epoxides. The B-norepoxide VIIb was shown to possess an α-configuration in the following manner. Upon treatment with boron fluoride etherate, the epoxide rearranged to yield a diol monoacetate VIIIb. The related diol VIIIa, obtained upon alkaline saponification of VIIIb, showed the high end absorption in the ultraviolet (ϵ_{205} 10,500) characteristic of a dioxocyclic double bond.[12] Upon oxidation, VIIIb yielded a non-conjugated unsaturated ketone IXb which possessed a single intense band in the infrared at 1730 cm.$^{-1}$, indicating the formation of a cyclopentanone whose absorption coincides with the

(1) For preliminary accounts of the results see, W. G. Dauben, G. A. Boswell, Jr., and G. H. Berezin, *J. Am. Chem. Soc.*, **81**, 6062 (1959), and W. G. Dauben, *Bull. soc. chim. France*, 1338 (1960).

(2) This work was supported, in part, by U. S. Public Health Grant CY-4284.

(3) General Electric Co. Fellow in Chemistry, 1958–1959.

(4) W. G. Dauben and G. J. Fonken, *J. Am. Chem. Soc.*, **78**, 4386 (1956).

(5) R. Willstätter and E. W. Mayer, *Ber.*, **41**, 2199 (1908); L. F. Fieser and M. Fieser, "Steroids," Reinhold Publishing Corp., New York, N. Y., 1959, p. 27.

(6) C. Djerassi, D. Marshall and T. Nakano, *J. Am. Chem. Soc.*, **80**, 4856 (1958).

(7) D. H. R. Barton, *J. Chem. Soc.*, 1027 (1953); W. G. Dauben, E. J. Blanz, Jr., J. Jiu and R. A. Micheli, *J. Am. Chem. Soc.*, **78**, 3752 (1956).

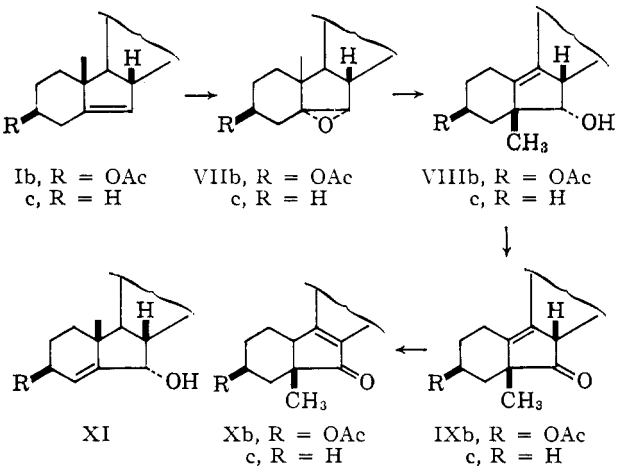
(8) We are indebted to Professor C. Djerassi for the determination of the optical rotatory dispersions.

(9) G. H. R. Summers, *J. Chem. Soc.*, 2908 (1959).

(10) J. Joska, J. Fajkos and F. Sôrm, *Coll. Czech. Chem. Comm.*, **25**, 2341 (1960).

(11) T. Goto and L. F. Fieser, *J. Am. Chem. Soc.*, **81**, 2276 (1959); T. Goto, *ibid.*, **82**, 2005 (1960).

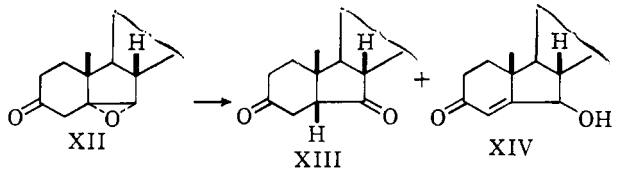
(12) P. S. Ellington and G. D. Meakins, *J. Chem. Soc.*, 697 (1960); R. A. Micheli and T. H. Applewhite, *J. Org. Chem.*, **27**, 345 (1962), and references contained therein.



band of the acetate group. Upon treatment with base, IXb rearranged to a conjugated ketone Xb. These results clearly indicate that in the attempted rearrangement of the epoxide the angular methyl group migrated from C-10 to C-5 since if such a migration had not occurred the diol monoacetate formed would have possessed structure XI and from such a structure no unconjugated ketone can be prepared. This migration of the 10β-methyl group is analogous to the formation of the well known Westphalen's diol from cholestane-3β,5α,6β-triol 3,6-diacetate[13] and thus clearly established that an α-epoxide had been formed in the B-nor case.

This finding of a methyl group migration in the reaction of the epoxide VIIb is of interest since with the related cholestane-5α,6α-epoxide only the expected migration of the C-6 β-hydrogen atom to form coprostane-6-one occurs.[14] Although the 3β-acetoxyl group in the B-nor-epoxide VIIb would not be expected to participate in the reaction, it could be remotely possible that by a change in the conformation of ring A such a grouping could become involved with a developing carbonium ion at C-5. The non-participation of the acetoxyl group was shown by preparation of the epoxide from Δ^5-B-norcholestene (Ic) and the finding of a similar rearrangement of the material to a 5β-methyl-19-nor steroid IXc upon reaction with boron trifluoride. It is suggestive that the migration of the angular methyl group in this B-nor case is a reflection of a strain inherent in the molecule which is relieved by the rearrangement, a strain which may be due to the *trans* fusion and/or the placement of the methyl group in a crowded position.

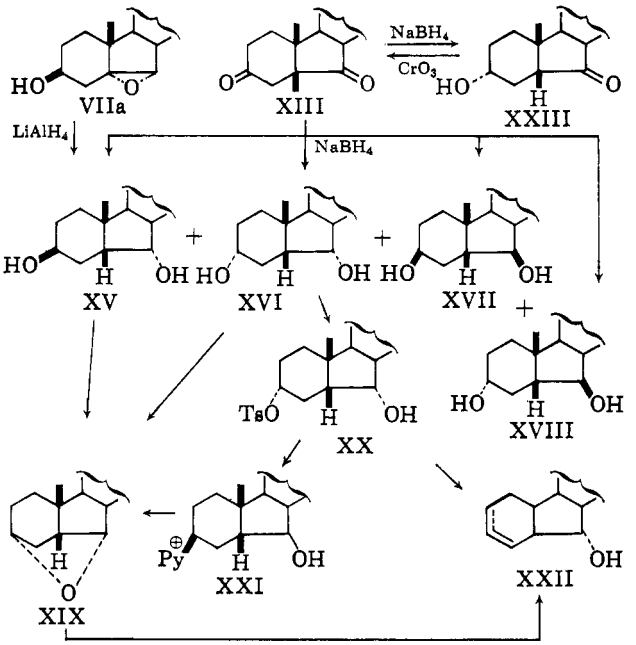
An insight into the sensitive energy balance of the B-nor steroid nucleus was gained by a study of the rearrangement of the related 3-keto-5α,6α-epoxide XII obtained from VIIb by hydrolysis and oxidation.



When the keto-epoxide XII was allowed to react with boron trifluoride etherate under the usual conditions two products were formed. The major product was B-norcoprostane-3,6-dione (XIII, Butenandt diketone) and the minor product was 6α-hydroxy-Δ^4-B-norcholestene-3-one (XIV). The structure of the latter material was established by its transformation by oxidation to the known Δ^4-B-norcholestene-3,6-dione. The formation of XIV rather than a compound in which the angular methyl group has rearranged is not surprising in view of the presence of the carbonyl function at C-3. The formation of the dione XIII, formed by migration of the 6β-hydrogen in preference to the C-10 methyl group, is unexpected and such a hydrogen migration must be yet another example of "long range conformational effects."[15] The diketone XII formed must be of the A/B *cis* series since the formation of a ketone upon rearrangement of an α-epoxide by boron trifluoride etherate has been shown to proceed with the stereospecific migration of the 6β-hydrogen atom.[14] This stereochemical result confirms the earlier assignment given to this diketone by Goto and Fieser[11] and by ourselves.[1]

In the reduction of B-norcoprostane-3,6-dione (XIII) with sodium borohydride Goto[11] reported the isolation of two of the four possible isomeric diols. On the basis of specific chemical transformations he established the configuration of the major diol formed as 3α,6α and the minor isomer was assigned the 3α,6β-configuration on the basis of reasonable but equivocal interpretation of the steric course of the hydride reaction. The borohydride reduction of the dione was also studied during the course of the present work and by chromatography of the hydride reduction mixture all four isomeric diols were obtained and the stereochemistry of the compounds was established.

The diol XV, an isomer not obtained by Goto,[11] was isolated in about 15% yield and was shown to possess the 3β,6α-configuration since it was identical with the diol prepared by lithium aluminum hydride reduction of 5α,6α-oxido-B-norcholestane-3β-ol acetate (VIIb). In this reduction of the epoxide the 3β,5α-diol also was formed. The diol XVI formed in 30%



yield was identical with the 3α,6α-diol previously described.[1,11] The two remaining isomers XVII and XVIII, obtained in 15% and 27%, respectively, must belong to the 6β-series. The 3α,6β configuration was assigned to XVIII since it was also formed along with XVI when 3α-hydroxycoprostane-6-one (XXIII)[16]

(13) T. Westphalen, *Ber.*, **48**, 1064 (1915); P. Bladon, H. B. Henbest and G. W. Wood, *J. Chem. Soc.*, 2737 (1952); B. Ellis and V. Petrow, *ibid.*, 2264 (1952).

(14) H. B. Henbest and T. I. Wrigley, *ibid.*, 4596 (1957).

(15) D. H. R. Barton, F. McCapra, P. J. May and F. Thudium, *ibid.*, 1297 (1960).

(16) W. G. Dauben, G. A. Boswell, Jr., W. Templeton and J. W. McFarland, *J. Am. Chem. Soc.*, in press.

was reduced with lithium aluminum hydride. Thus, the remaining isomer XVII must be assigned the 3β,6β-structure.

The reactions of these diols with *p*-toluene- or benzenesulfonyl chloride are most remarkable and Goto and Fieser[11] have found that both the 3α,6α-(XVI) and the 3α,6β-(XVIII) diols upon heating in pyridine with the acid chloride were converted to the same 3α,6α-epoxide XIX. When the reaction was conducted at zero degrees, XVI was transformed into the 3α-monotosylate XX and XVIII into the 3α,6β-ditosylate. It has now been found that reaction of the 3β,6α-diol at zero degrees yielded only the 3α,6α-epoxide XIX. The formation of the epoxide from XVI must proceed *via* the monotosylate XXI by displacement of the tosylate grouping by pyridine to yield the 3β-pyridinium derivative XXI and this latter grouping then must be displaced by the 6α-hydroxyl group. In line with this proposal was the finding that XX upon reaction with pyridine was converted to the ether but when XX was allowed to react with potassium *tert*-butoxide only the unsaturated alcohol XXII was formed. This alcohol was identical with the product formed by acid-catalyzed cleavage of the 3α,6α-epoxide.

Experimental[17]

B-Norcoprostane-3α-ol (VI).—To a solution of 0.35 g. (0.94 mmole) of B-norcoprostanone in 30 ml. of ether, there was added a solution of excess lithium aluminum hydride in anhydrous ether. The reaction mixture was allowed to stir at room temperature for 4 hours, the excess reducing agent was decomposed by cautious addition of water, and the product isolated with ether in the usual manner. Evaporation of the ether under reduced pressure yielded 0.34 g. of an oil which slowly solidified. The infrared spectrum of this crude product showed that the product was a mixture of about 75% of the 3α-isomer and 25% of the 3β-isomer. The total crude product was chromatographed on 10 g. of Woelm neutral alumina (Act. III). Elution with petroleum ether and petroleum ether–benzene (2:1) returned 0.22 g. (63%) of crystalline material which upon recrystallization from methanol (slowly) yielded 0.21 g. of B-norcoprostane-3α-ol as fine, thread-like needles, m.p. 95–96° (sealed capilliary), $[\alpha]^{20}D$ +16°.

Anal. Calcd. for $C_{26}H_{46}O$ (374.63): C, 83.35; H, 12.38. Found: C, 83.21; H, 12.10.

Further elution with petroleum ether–benzene (2:1) returned 0.105 g. (30%) of oily material which crystallized slowly. The infrared spectrum of the material showed it to be a mixture of the two isomers.

B-Norcoprostane-3β-ol (II) and B-Norcholestane-3β-ol (III).—A solution of 1.49 g. (3.6 mmoles) of B-norcholesteryl acetate in 50 ml. of glacial acetic acid containing 0.25 g. of platinum oxide was hydrogenated at atmospheric pressure and room temperature; the hydrogen uptake ceased after 30 minutes. The catalyst was removed by filtration, the filtrate evaporated under reduced pressure, and the residual clear oil saponified by heating under reflux with 50 ml. of 5% methanolic potassium hydroxide. The solution was diluted with water, the suspension extracted with ether, the ethereal solution washed with water and dried, and the solvent removed. The crystalline residue (1.32 g.) was chromatographed on Woelm neutral alumina (Act. III).

Elution with petroleum ether–benzene (1:1) gave 0.773 g. (74%) of B-norcoprostane-3β-ol (II), m.p. 50–56°. Recrystallization from methanol gave needles, m.p. 55–56°, yield 0.693 g. (66%). This material after drying for 2 days at room temperature and 3×10^{-3} mm. melts at 75–77°. A sample was sublimed to give material melting at 74–76°, $[\alpha]^{25}D$ +16° (lit.[4] m.p. 77.5–78.2°, $[\alpha]^{21}D$ +14.5°).

Further elution with benzene gave 0.167 g. (16%) of a mixture of isomers. Continued elution with benzene yielded 0.162 g. (16%) of needles, m.p. 118–120°. Recrystallization from methanol gave product, m.p. 132–133°, $[\alpha]^{20}D$ +8°.

Anal. Calcd. for $C_{26}H_{46}O$ (374.63): C, 83.35; H, 12.38. Found: C, 83.33; H, 12.09.

B-Norcholestane-3-one (V).—A solution of 0.11 g. (0.29 mmole) of B-norcholestane-3β-ol, 0.10 g. of sodium dichromate dihydrate and 10 ml. of glacial acetic acid was warmed on the steam-bath for 10 minutes, the green solution diluted with ice-water, and extracted with ether. The ethereal extract was washed with water, sodium bicarbonate solution, and dried over magnesium sulfate. The ether was evaporated at reduced pressure and the crude product (0.085 g., m.p. 75–80°) recrystallized twice from methanol; yield 0.042 g. (38%), m.p. 96–97°, $[\alpha]^{20}D$ +36°; R.D. in methanol solution (*c* 0.172): $[\alpha]_{589}$ +40 ,$[\alpha]_{312}$ +1200, $[\alpha]_{265}$ −1500.

Anal. Calcd. for $C_{26}H_{44}O$ (372.61): C, 83.80; H, 11.90. Found: C, 83.66; H, 12.06.

B-Norcoprostane-3-one (IV).—A solution of 0.95 g. (2.5 mmoles) of B-norcoprostane-3α-ol, 0.38 g. of sodium dichromate dihydrate and 10 ml. of glacial acetic acid was warmed on a steam-bath for 15 minutes and processed in the usual manner. The crude product was recrystallized twice from methanol; yield 0.75 g. (81%), m.p. 75–76°, $[\alpha]^{20}D$ +18° (lit.[4] m.p. 62.6–63.7°, $[\alpha]^{20}D$ +19°); R.D. in methanol solution (*c* 0.162): $[\alpha]_{589}$ +21, $[\alpha]_{310}$ −580, $[\alpha]_{265}$ +1280.

5α,6α-Oxido-B-norcholestane-3β-ol Acetate (VIIb).—To a solution of 2.0 g. (0.48 mmole) of B-norcholesteryl acetate in 20 ml. of dry ether there was added 2.0 g. of monoperphthalic acid (determined by titration) in 20 ml. of ether. The reaction was allowed to stand at room temperature for 15 hours, then diluted with water and extracted with ether. The ether extracts were washed with water, saturated sodium bicarbonate solution, and saturated sodium chloride solution. The solvent was evaporated under reduced pressure and the residue recrystallized twice from methanol; yield 1.85 g. (89%), m.p. 107–108°, $[\alpha]^{20}D$ −32°.

Anal. Calcd. for $C_{28}H_{46}O_3$ (430.65): C, 78.09; H, 10.77. Found: C, 78.11; H, 10.88.

A solution of the acetate (2.0 g., 4.65 mmoles) in 50 ml. of 5% methanolic potassium hydroxide was heated under reflux for 1 hour. Upon cooling, the solution set to a solid mass of white crystals which was removed and the filtrate diluted with water and extracted with ether to yield additional material. The combined products were recrystallized from acetone to yield 1.66 g. (93%) of 5α,6β-oxido-B-norcholestane-3β-ol, m.p. 139–140°.

Anal. Calcd. for $C_{26}H_{44}O_2$ (388.61): C, 80.35; H, 11.41. Found: C, 80.63; H, 11.49.

19-Nor-5β-methyl-$\Delta^{9(10)}$-B-norcholestene-3β,6α-diol 3-Acetate (VIIIb).—A solution of 0.56 g. (1.3 mmoles) of epoxide VIIb, 0.50 ml. of redistilled boron trifluoride etherate and 10 ml. of dry benzene was allowed to stand for 12 hours at room temperature. The solution was washed with 5% sodium bicarbonate solution, dried, and the benzene removed. The residue was chromatographed on Woelm neutral alumina (Act. III).

Elution with petroleum ether gave a yellow oil (0.22 g.) which solidified on standing and was recrystallized from acetone containing a few drops of water; m.p. 146–147°. The product was not investigated further.

Further elution with petroleum ether–benzene (1:1) and benzene yielded 0.286 g. (51%) of colorless crystals, m.p. 90–93°. The crude product was recrystallized from aqueous acetone to yield VIIIb, 238 mg. (42%), m.p. 102–103°, $[\alpha]^{20}D$ +60°.

Anal. Calcd. for $C_{28}H_{46}O_3$ (430.65): C, 78.09; H, 10.77. Found: C, 78.13; H, 10.88.

19-Nor-5β-methyl-$\Delta^{9(10)}$-B-norcholestene-3β,6α-diol (VIIIa).—A solution of 0.122 g. (0.28 mmole) of the acetate VIIIb in 20 ml. of 5% ethanolic potassium hydroxide was allowed to stand overnight at room temperature. The reaction mixture was processed in the usual fashion and the crude product recrystallized from acetone containing a few drops of water; yield 0.087 g. (80%), m.p. 135–138°, ϵ_{205} 10,700.

Anal. Calcd. for $C_{26}H_{44}O_2$ (388.61): C, 80.35; H, 11.41. Found: C, 80.83; H, 11.34.

19-Nor-5β-methyl-$\Delta^{9(10)}$-B-norcholestene-3-ol-6-one 3-Acetate (IXb).—To a solution of 183 mg. (0.425 mmole) of 3-acetoxy-6-ol VIIb in 50 ml. of purified acetone there was added, over a period of 1 minute with stirring and in a nitrogen atmosphere, 0.15 ml. of a 2.67 *M* solution of chromic acid in sulfuric acid.[18] Stirring was continued for 4 minutes, 2 ml. of methanol added and the product isolated with ether. The crude product was recrystallized from methanol; yield 130 mg. (70%), m.p. 123.0–124.0°, $[\alpha]^{26}D$ +71°; λ_{max} 298 mμ (ε 36), ϵ_{231} 235, ν_{max} 1730 cm.$^{-1}$ (cyclopentanone and ester).

Anal. Calcd. for $C_{28}H_{44}O_3 \cdot 0.5H_2O$ (437.64): C, 76.84; H, 10.36. Found: C, 76.55; H, 10.35.

A solution of 10 mg. of the ketone in 25 ml. of 5% methanolic potassium hydroxide was allowed to stand at room temperature for 15 hours. The usual work-up yielded 8 mg. of an oil, λ_{max} 245 mμ (ε 11,000); ν_{max} 3380 cm.$^{-1}$ (hydroxyl), 1730 cm.$^{-1}$ (very weak band), 1690 cm.$^{-1}$ (conj. five-ring ketone), 1645 cm.$^{-1}$ (conj. olefin).

(17) All melting points are corrected. All optical rotations were determined in chloroform. All infrared spectra were taken in carbon disulfide unless noted. Analyses were performed by the Microanalytical Laboratory, College of Chemistry, University of California.

(18) K. Bowden, I. M. Heilbron, E. R. H. Jones and B. C. L. Weedon, *J. Chem. Soc.*, 39 (1946); C. Djerassi, R. R. Engle and A. Bowers, *J. Org. Chem.*, **21**, 1547 (1956).

Δ^5-B-Norcholestene.—A solution of 2.0 g. (5.37 mmoles) of B-norcholesterol in 10 ml. of purified thionyl chloride was allowed to stand at room temperature for 2 hours and the excess thionyl chloride removed under reduced pressure. The crude 3-chloro derivative was recrystallized from acetone to yield 1.5 g. of material which was immediately dissolved in 50 ml. of isoamyl alcohol. The solution was then heated to reflux temperature, 3.0 g. of sodium added, and the mixture allowed to reflux for 1 hour. The reaction mixture was poured into water, the product extracted with ether, the ether washed with hydrochloric acid and sodium bicarbonate solution, and dried. The solvent was removed under reduced pressure and the crude Δ^5-B-norcholestene was recrystallized from aqueous acetone; yield 1.2 g. (63%), m.p. 63–64°, $[\alpha]^{25}D$ −129°.

Anal. Calcd. for $C_{26}H_{44}$ (356.61): C, 87.56; H, 12.44. Found: C, 87.27; H, 12.26.

5α,6α-Oxido-B-norcholestane (VIIc).—An ethereal solution of 175 mg. (0.49 mmole) of Δ^5-B-norcholestene containing one equivalent of monoperphthalic acid was allowed to stand at 0° for 18 hours. The solution was washed with sodium bicarbonate, dried, and evaporated. The residue was chromatographed on Woelm neutral alumina (Act. III) and the epoxide was eluted with petroleum ether. The crude product was recrystallized from acetone; yield 50 mg. (27%), m.p. 97–98°, $[\alpha]^{25}D$ −41°.

Anal. Calcd. for $C_{26}H_{44}O$ (372.61): C, 83.30; H, 11.90. Found: C, 83.65; H, 11.76.

19-Nor-5β-methyl-$\Delta^{9(10)}$-B-norcholestene-6α-ol (VIIIc).—A solution of 185 mg. (0.5 mmole) of 5α,6α-oxido-B-norcholestane and 0.25 ml. of freshly distilled boron trifluoride etherate in 5 ml. of dry benzene was allowed to stand at room temperature for 12 hours. The solution was poured into water, extracted with ether, the extracts were washed and dried and the solvent was evaporated at reduced pressure. The residue was chromatographed on Woelm neutral alumina (Act. III). Elution with petroleum ether and petroleum ether–benzene (9:1) yielded the product which was recrystallized from acetone; yield 130 mg. (70%), m.p. 104–105°, $[\alpha]^{25}D$ +74°, ϵ_{205} 4,700.

Anal. Calcd. for $C_{26}H_{44}O$ (372.61): C, 83.30; H, 11.90. Found: C, 83.52; H, 11.71.

19-Nor-5β-methyl-$\Delta^{8(9)}$-B-norcholestene-6-one (Xc).—A solution of 70 mg. (0.18 mmole) of the 6α-ol VIIIc in 10 ml. of purified acetone was oxidized with 0.075 ml. of 2.67 *M* solution of chromic acid in sulfuric acid[18] as described for the preparation of IXb. The crude reaction product had the following spectral properties: ν_{max} 1740, 1665 cm.$^{-1}$; ϵ_{205} 2,330, ϵ_{200} 5,200.

The unconjugated ketone was allowed to stand for 14 hours at room temperature with 20 ml. of 10% potassium hydroxide in methanol. After dilution with water and extraction with ether there was obtained 55 mg. of a yellow solid which was chromatographed on Woelm neutral alumina (Act. III). Elution with petroleum ether and petroleum ether–benzene (9:1) gave 15 mg. of the conjugated enone which was recrystallized from methanol; m.p. 79–80°, $[\alpha]^{25}D$ −36.8°, λ_{max} 245 mμ (ϵ 11,000); $\nu_{max}^{CHCl_3}$ 1690, 1632 cm.$^{-1}$.

Anal. Calcd. for $C_{26}H_{42}O$ (370.60): C, 84.26; H, 11.42. Found: C, 84.17; H, 11.68.

5α,6α-Oxido-B-norcholestane-3-one (XII).—A solution of 290 mg. (0.75 mmole) of 5α,6α-oxido-B-norcholestane-3β-ol (VIIa) in 25 ml. of pure dry acetone was cooled to −25° and stirred under nitrogen for 15 minutes with 0.25 ml. of a 2.67 *M* solution of chromic acid in sulfuric acid.[18] Excess methanol was added to destroy excess oxidizing reagent and the product extracted with ether. The ether was evaporated under reduced pressure and the residue recrystallized from ethanol; yield 145 mg. (50.3%), m.p. 157–160°, $[\alpha]^{20}D$ +12°.

Anal. Calcd. for $C_{26}H_{42}O_2$ (386.60): C, 80.77; H, 10.95. Found: C, 80.60; H, 10.88.

Rearrangement of 5α,6α-Oxido-B-norcholestane-3-one (XII) with Boron Trifluoride.—A solution of 1.0 g. (2.59 mmoles) of keto-oxide XII and 1 ml. of redistilled boron trifluoride etherate in 50 ml. of dry benzene was allowed to stand at room temperature for 18 hours, the solution poured into an aqueous solution of sodium bicarbonate, the benzene layer separated, and the solvent evaporated under reduced pressure. The residue (1.0 g.) was chromatographed on Woelm neutral alumina (Act. III). Elution with benzene gave 450 mg. (45%) of B-norcoprostane-3,6-dione (XIII), m.p. 110–111° (mixture m.p. and comparison infrared spectrum identical with authentic sample). Elution with 9:1 and 4:1 benzene–ether yielded 300 mg. (30%) of Δ^4-B-norcholestene-3-one-6α-ol (XIV). The compound was recrystallized from acetone; m.p. 164–165°, γ_{max}^{EtOH} 241 mμ (ϵ 14,500).

Anal. Calcd. for $C_{26}H_{42}O_2$ (386.60): C, 80.77; H, 10.95. Found: C, 80.52; H, 10.69.

A solution of 7.5 mg. of hydroxyenone XIV in 5 ml. of acetone was stirred for 15 minutes at 0° with 0.015 ml. of a 2.67 *M* solution of chromic acid in sulfuric acid.[18] The product was isolated in the usual fashion and the pale yellow needles melted at 113–115°, no depression upon admixture with authentic Δ^4-B-norcholestene-3,6-dione[4]; the infrared spectra of the two samples were identical.

Reduction of B-Norcoprostane-3,6-dione with Sodium Borohydride.—A solution of 1.57 g. (4.05 mmoles) of B-norcoprostane-3,6-dione in 30 ml. of methanol and 20 ml. of ether was warmed to 50°. To this solution there was added 1.0 g. of sodium borohydride with stirring. The solution was stirred for 3 hours during which time the temperature was allowed to fall to 25°. The reaction mixture was decomposed with dilute hydrochloric acid and processed in the usual manner. The crude product was chromatographed on 60 g. of Woelm neutral alumina (Act. III).

Elution with petroleum ether–benzene (1:1) gave 0.029 g. of starting dione. Continued elution with petroleum ether–benzene (1:9) afforded 0.47 g. (30%) of a crystalline diol, m.p. 130–135°. The material was recrystallized from acetone; m.p. 140–142°, $[\alpha]^{20}D$ ±0° and was identical with the 3α,6α-isomer. Upon oxidation, the diol yielded starting dione.

Anal. Calcd. for $C_{26}H_{46}O_2$ (390.63): C, 79.94; H, 11.87. Found: C 79.61; H, 11.57.

Elution with benzene gave 0.328 g. (21%) of crystalline material which crystallized from acetone as a mixture of needles and square plates. The material was a mixture of the 3α,6α-diol and the 3β,6α-diol. Elution with benzene–ether (1:1) gave 0.212 g. (14%) of long needles, m.p. 80–85°. Recrystallization from aqueous acetone yielded the 3β,6α-diol as a stable hydrate, m.p. 75–85°. Slow recrystallization from acetone gave long silky needles, m.p. 85–110°, while recrystallization from nitroethane gave a crystalline form melting at 137–141°. The sample was proved to be homogeneous by paper chromatography (under conditions which separate all four isomers). Oxidation of the diol yielded starting dione.

Anal. Calcd. for $C_{26}H_{46}O_2 \cdot 0.5H_2O$ (399.63): C, 78.15; H, 11.87. Found: C, 77.90; H, 11.98.

Elution with ether afforded 0.42 g. (27%) of the 3α,6β-diol. The material, m.p. 170–173°, recrystallized from acetone, was identical with an authentic sample.[16] Upon oxidation the diol yielded starting dione.

Washing the column with ether–methanol (1:1) returned 0.130 (15%) of crystalline 3β,6β-diol. The compound recrystallized from aqueous acetone in the form of long needles, m.p. 80–85°, which is a hydrate. Recrystallization from dry acetone afforded needles, m.p. 125–127°.

Anal. Calcd. for $C_{26}H_{46}O_2 \cdot 0.5H_2O$ (399.63): C, 78.15; H, 11.87. Found: C, 78.25; H, 11.99.

Anal. Calcd. for $C_{26}H_{46}O_2$ (390.63): C, 79.94; H, 11.87. Found: C, 79.97; H, 11.90.

Reduction of 5α,6α-Oxido-B-norcholestane-3β-ol Acetate (VIIb).—A solution of 3.1 g. of the oxido-acetate VIIb in 100 ml. of dry ether was allowed to react at room temperature for 48 hours with 3 g. of lithium aluminum hydride. The mixture was treated with methanol, then with ammonium chloride solution, the ether layer separated, and dried. Removal of the solvent gave 2.3 g. of a mixture of the 3,5- and 3,6-diols.

The mixture was dissolved in 10 ml. of acetic anhydride and 10 ml. of dry pyridine and allowed to stand for 30 hours at room temperature. After removal of the solvents, the residue was dissolved in ether and the ethereal solution washed with dilute hydrochloric acid, water, and sodium bicarbonate solution. After drying and removal of solvent, the material was chromatographed on Woelm neutral alumina (Act. III). Elution with petroleum ether gave 0.56 g. of a diacetate and elution with ether gave 0.94 g. of a monoacetate. The material eluted with petroleum ether was treated with a large excess of lithium aluminum hydride and processed in the usual manner. The 440 g. of white crystals, m.p. 75–85°, was identical in all regards with the 3β,6α-diol prepared above.

Reaction of B-Norcoprostane-3β,6α-diol with *p*-Toluenesulfonyl Chloride at 0°.—To a well stirred solution of 350 mg. (0.9 mmole) of B-norcoprostane-3β,6α-diol in 4 ml. of dry pyridine at 0° there was added 400 mg. of *p*-toluenesulfonyl chloride. The solution was allowed to stand at 0° for 12 hours, then poured into 100 ml. of ice-water and the reaction mixture extracted with ether. The ethereal solution was washed with two 50-ml. portions of 2 *N* hydrochloric acid and then equal quantities of sodium bicarbonate solution. The ethereal solution was dried over magnesium sulfate and the solvent removed under reduced pressure to yield 320 mg. of an oil which crystallized when triturated with acetone. The product was recrystallized from aqueous acetone; yield 286 mg. (85%), m.p. 84.5–86.0°. The product had an infrared spectrum identical with B-norcoprostane-3α,6α-oxide (XIX) and upon admixture with an authentic sample no depression in m.p. was noted.

Δ^3-B-Norcoprostene-6α-ol (XII). (a) From the Oxide XIX.—A solution of 501 mg. (1.34 mmoles) of B-norcoprostane-3α,6α-oxide (XIX) and 545 mg. of *p*-toluenesulfonic acid in 16 ml. of acetic anhydride was heated on a steam-bath for 5 hours. The dark red solution was poured into 150 ml. of water and the acid

neutralized with solid sodium bicarbonate. The product was extracted with ether, the ethereal solution dried and the solvent removed. The residue was chromatographed on 18 g. of Woelm neutral alumina (Act. III). Elution with pentane gave a crude ester fraction which was saponified with 5% potassium hydroxide in methanol. The resulting alcohol was chromatographed on 5 g. of Woelm neutral alumina and elution with petroleum ether followed by crystallization from acetone yielded 40 mg. (8%) of XII, m.p. 102–103°, $[\alpha]^{27}D$ +2°.

Anal. Calcd. for $C_{26}H_{44}O$ (372.61): C, 83.31; H, 11.90. Found: C, 83.53; H, 11.78.

(b) **From B-Norcoprostane-3α,6α-diol 3-Monotosylate (XX).**—A solution of 150 mg. (0.275 mmole) of XX[11] in 50 ml. of dry *tert*-butyl alcohol containing 2.3 g. of potassium *tert*-butoxide was heated at 50° for 4 hours. The solution was allowed to stand at room temperature overnight, neutralized with glacial acetic acid, and concentrated under reduced pressure to a small volume. The remaining solution was poured into water and extracted with ether. After removal of the ether the solid residue was recrystallized from acetone containing a few drops of water; yield 67 mg. (65%), m.p. 102–103°, no depression on admixture with material prepared above.

Reaction of B-Norcoprostane-3α,6α-diol 3-Tosylate (XX) with Pyridine.—A solution of 85 mg. (0.16 mmole) of XX in 4 ml. of pyridine was heated at 90° for 40 minutes. The product was isolated in the usual manner and chromatographed on Woelm neutral alumina (Act. I). Elution with petroleum ether yielded 36 mg. (58%) of B-norcoprostane-3α,6α-oxide (XIX), m.p. 82.0–85.0°.

[CONTRIBUTION FROM THE DIVISION OF LABORATORIES AND RESEARCH, NEW YORK STATE DEPARTMENT OF HEALTH, ALBANY, AND THE DEPARTMENT OF BIOCHEMISTRY, ALBANY MEDICAL COLLEGE, ALBANY, N. Y.]

Studies of Completely Deuteriated Proteins. I. The Immunochemistry of the Deuteriated Protein and its Hydrogen Analog

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RECEIVED FEBRUARY 8, 1963

Qualitative and quantitative immunochemical evidence, double diffusion studies and precipitin curves are presented to establish the identity of a deuterio protein and its hydrogen analog in primary, secondary and tertiary protein structure.

The isolation and characterization of a completely deuteriated algal protein from blue-green algae have been reported by Berns, *et al.*[1] The physical and chemical properties described indicate that deuterio and hydrogen proteins are quite likely chemically identical and physically similar although isotopically different. The question of whether there are significantly different primary, secondary, tertiary and quaternary structural characteristics in the deuterio protein and its hydrogen analog may be answered in part but not entirely by the extremely sensitive techniques of immunochemistry. Certain positive conclusions concerning structural features of the protein can be reached and these are of considerable importance in determining the validity of additional investigations of the physical chemistry of deuterio[2] and protio proteins and the applicability of this system to elucidating the role of hydrogen bonding and possibly internal rotation in protein structure. It must be established first that the proteins are in fact the same protein except for isotopic differences and that the isotopic substitution has not altered the over-all protein structure so that comparison of the physical and chemical behavior of the systems is meaningless, since the so-called deuterio and hydrogen proteins are in actual fact different proteins.

Experimental

Materials.—All samples of phycocyanin were isolated and purified from algal cultures as described by Berns, *et al.*[1] In all experiments the protein samples were dialyzed into 0.15 *M* saline previous to use.

Methods.—Ouchterlony plates and Oudin tubes were set up as described by Kabat and Mayer,[3] and the precipitin procedure used was similar to that of these authors.[3] The amount of nitrogen in the precipitates was determined with a micro-Kjeldahl procedure similar to the Markham modification.[3] Rabbits were inoculated with a suspension of phycocyanin in complete Freund's adjuvant. The suspension was equal volumes of adjuvant[4] and protein solution. Each rabbit was injected with about 3 mg. of protein. Approximately 0.3 ml. of suspension was injected into each toe pad and 0.4 ml. subcutaneously in the back of the neck. Five rabbits were injected with deuterio phycocyanin isolated from *Plectonema calothricoides* and five with the protio protein. One month later the injections were repeated, the animals rested for one week, and then approximately 50 ml. of blood was collected from each rabbit by cardiac puncture. The animals were rested for a month, reinoculated and one week later bled again.

First course antisera to the deuterio phycocyanin from *P. calothricoides* and also to the corresponding protio protein were diluted 1:5 and 1 ml. of this dilution was added to 1 ml. of serially diluted antigen. Both antisera and antigen preparations were clarified previous to their use. All precipitin procedures were done at constant volume and at the antiserum dilution. The precipitins were then allowed to stand for 3 days at 1°. Appropriate antigen and antiserum blanks were done simultaneously. The precipitates were spun down and washed[3] and transferred to Kjeldahl flasks for nitrogen determination. The supernatants were saved and checked for the presence of antibody and antigen. In all the experiments reported in this study the results of the tests of supernatant for excess antibody and antigen were in general agreement with the information derived from the determination of the amount of nitrogen in the precipitates. Precipitin experiments were also carried out with crude algal extracts. An antigen concentration of about 0.2% was used in the agar diffusion experiments.

The precipitin lines on Ouchterlony plates were examined for fluorescence with a long wave length, ultraviolet mineral light. Sedimentation values for the deuterio phycocyanin from *P. calothricoides* and for the protio phycocyanin from the same source, both in 1% saline solution, were determined by a Spinco model E analytical ultracentrifuge. Both proteins were sedimented in the same run in which one cell was used with a quartz 1° positive wedge window.

Results

I. Diffusion Studies.—First course antisera in agar, in Oudin tubes against which the protio and deuterio antigen diffused, exhibited definite precipitin lines. Sharp single lines were observed in the antisera prepared against protio protein. The antideuterio antisera in Oudin tubes exhibited multiple lines. Double diffusion Ouchterlony plates were then set up in which antideuterio *P. calothricoides* antisera were allowed to diffuse simultaneously against deuterio and protio phycocyanin from *P. calothricoides*, deuterio phycocyanin from *Phormidium luridum* and protio phycocyanin from *Synechococcus lividus*.[5] Antisera to protio phycocyanin were allowed to diffuse against the same antigens. The results of this double diffusion and other

(1) D. S. Berns, H. Crespi and J. J. Katz, *J. Am. Chem. Soc.*, **85**, 8 (1963).

(2) The term "deuterio protein" refers to the protein with deuterium substituted for hydrogen in all normally non-exchangeable positions. "Protio protein" is the normal hydrogen-containing protein.

(3) E. Kabat and M. Mayer, "Experimental Immunochemistry," C. C. Thomas, Springfield, Ill., second edition, 1961, pp. 22–96. Note that antigens used in the Ouchterlony plates were of less purity than those used in immunizing.

(4) Prepared by C. Brown of this Laboratory.

(5) A culture of this alga was kindly supplied by D. L. Dyer, Martin Co., Denver, Colo.