with about 3.5 liters of acetone to remove impurities. Successful removal of impurities was indicated by the fact that crystalline cholic acid could be obtained easily from the last 50 ml. of acetone wash by evaporation to dryness and scratching. (The rate of both charging and washing the column was maintained at not greater than 3 ml. per min.) The methyl cholate was then eluted with about 2.5 liters of absolute methanol. This eluate was evaporated to 100 ml., seeded with pure methyl cholate and crystallized at 5° for 24 hours. The filtered and dried crystals (4.5 g.) were saponified by dissolving in 100 ml. of 0.8 *M* aqueous sodium hydroxide solution and heating for one hour on the steam-bath. This solution was acidified with 50 ml. of 2.5 *N* hydrochloric acid and, after addition of 5 g. of sodium sulfate, was extracted with an equal volume of a mixture containing one part isopropyl alcohol to three parts ethyl ether. The non-aqueous phase was treated as the previous similar solution of crude material, *i.e.*, washed, dried over anhydrous sodium sulfate and evaporated to dryness. Final crystallization of the residue from acetone yielded 3.6 g. of deuterocholic acid containing 20.0 atom per cent. deuterium, m.p. 199–204°. On the basis of frontal analysis, we believe the purity of this material to be above 97%; we attribute the wide melting point range to polymorphism.

Analysis for deuterium content was carried out by combustion of the steroid in a stream of dried oxygen and measurement of the infrared absorption[6,7] of the condensed water of combustion at 3.198 μ, diluting if necessary with ordinary water to bring the deuterium oxide concentration into measurable range. Infrared measurements were made with a model 12C Perkin–Elmer infrared spectrophotometer.

Our interest in these exchange reactions has arisen from the need for stably labelled materials in quantitative analysis by the isotope dilution method.[8,9]

In the light of the findings of van Heyningen, Rittenberg and Schoenheimer[10] concerning the hydrogen–deuterium exchange in the fatty acids under the influence of alkali and platinum catalyst, it seems reasonable to assume that a large proportion of the hydrogen atoms exchanged with the bile acid takes place along the fatty acid side chain. There is evidence, however, which we have obtained since the subject matter of this paper was completed which indicates that some of the observed exchange takes place on the steroid polycyclic nucleus probably at positions alpha to the oxygen-bonded carbon atoms (exclusive of the spontaneously labile hydrogens of the hydroxyl groups). In any case whatever their positions all the deuterium atoms introduced are relatively stable ones since they have been demonstrated not to be labile in the absence of the platinum catalyst.

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The Conversion of 17α,21-Dihydroxypregnane-3,20-dione to 3α,17α,21-Trihydroxypregnan-20-one *in vitro*[1,2]

BY FRANK UNGAR AND RALPH I. DORFMAN

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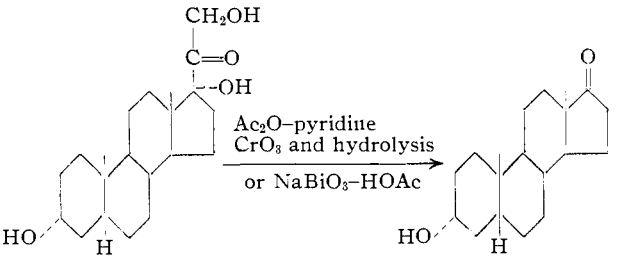
A possible intermediate in the metabolism of Reichstein's substance S, 17α,21-dihydroxypregnane-3,20-dione (dihydro S), has been administered to a female rheumatoid arthritic patient, and 3α-hydroxyetiocholan-17-one, 3α,17α,20α-pregnanetriol and 3α,17α,21-trihydroxypregnan-20-one (tetrahydro S) have been isolated from the urine.[3] The diverse reactions indicated by the *in vivo* study have been investigated in more detail using various *in vitro* systems. This report is concerned specifically with the conversion of dihydro S to the tetrahydro derivative, 3α,17α,21-trihydroxypregnan-20-one, by incubation with a rat liver homogenate.

The tetrahydro S obtained from the urine following the administration of dihydro S *in vivo* was identical to the product of the incubation with rat liver homogenate. The product of the incubation was detected, using the paper chromatographic technique,[4] with the triphenyltetrazolium chloride reagent. The compound migrated 0.12–0.17 cm./hr. in the toluene–propylene glycol system and the dihydro S starting material migrated 1.2–1.5 cm./hr. The structure of the tetrahydro S was established by the data given in Fig. 1. Chromic acid



3α,17α,21 - Trihydroxypregnan - 3α - Hydroxyetiocholan - 17 - 20-one: free, m.p. 214–216°; $[\alpha]^{27}D$ +60° (ethanol); one: diacetate, m.p. 201–206°, $[\alpha]^{27}D$ +77° (ethanol)

	alc. M_D	ΔM_D
3α,5β-THS diacetate	+338°	+126°
3α,5β-THS free	+212°	
3β,5β-THS diacetate	+228°	+38°
3β,5β-THS free	+190°	

Fig. 1.

oxidation of the tetrahydro S diacetate followed by hydrolysis, and oxidation of the tetrahydro S with $NaBiO_3$, both yielded 3α-hydroxyetiocholan-17-one. A comparison of the molecular rotational differences (ΔM_D) of the free 3α,5β-tetrahydro S and its diacetate with that of the free 3β,5β-tetrahydro S and its diacetate, showed a large positive increase for the 3α- as compared to the 3β-derivative.[5] The reduction of the 3-ketone to the 3α-hydroxy group with rat liver homogenate proceeded to the extent of 60–70%. In contrast, only minute amounts of tetrahydro S were isolated from the urine in the *in vivo* study. Dihydro S was converted to the 3α,5β-tetrahydro S by incubation with rabbit liver homogenate and rabbit kidney homogenate, the latter conversion proceeding to a lesser extent. The reduction to the 3α,5β-tetrahydro S with rat liver slices also proceeded to the extent of 60%. In this case, also, a small amount of the 3β,5β-tetrahydro S (0.5%) was isolated.

The 3α-hydrogenase of rat liver has been charac-

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terized further. The major activity resides in a cell-free supernatant of a phosphate buffer extract, *p*H 7.4, of rat liver ground in a meat grinder. The activity was retained in the supernatant of an acetone powder of the ground rat livers. Dialysis of this supernatant lowered the conversion of the 3-ketone to the 3α-hydroxy group to 30%, but with the addition of 0.002 *M* DPN, the conversion increased to 95%. Further studies of this reaction are in progress.

Experimental[6]

Hydrolysis of 21-Acetoxy-17α-hydroxypregnane-3,20-dione.—Three grams of 21-acetoxy-17α-hydroxypregnane-3,20-dione was hydrolyzed with $KHCO_3$ in methanol at room temperature for 24 hours to yield 2.74 g. of white crystalline material, m.p. 185–195°. The material was chromatographed on a column containing 90 g. of silica gel and eluted with benzene, benzene–ethyl acetate mixtures and methanol. Elution with benzene ethyl acetate (2:1) yielded 2.3 g. of crystalline material which was recrystallized from acetone and ethanol to give crystals melting at 200–202°, $[\alpha]^{27}D$ +62° (ethanol).

Incubation of 17α,21-Dihydroxypregnane-3,20-dione (Dihydro S) with Rat Liver Homogenate.—The livers of adult male albino rats were quickly removed following decapitation and homogenized for 20 sec. in a Waring blender in 0.1 *M* phosphate buffer, *p*H 7.4. A total of 820 mg. of dihydro S was incubated (10 mg. of steroid per 10 g. equivalent of tissue wet weight) by shaking in air at 38° in a Warburg for 1.5 hours. Following acetone precipitation of the proteins, and a 70% methanol–petroleum ether partition to remove the lipids, the crude extract (2.77 g.) was chromatographed on a column containing 170 g. of silica gel.

Isolation of 3α,17α,21-Trihydroxypregnan-20-one.—The various fractions of the silica gel chromatogram were eluted with a system of benzene, benzene–ethyl acetate mixtures, ethyl acetate and methanol. Elution with benzene–ethyl acetate (2:1) yielded a total of 660 mg. of crystalline product. Aliquots of the various eluates were spot-checked by paper partition chromatography using the toluene–propylene glycol systems for four hours. The dihydro S and tetrahydro S zones were visualized with the triphenyltetrazolium chloride reagent.

Recrystallization of the pooled homogeneous fractions yielded 389 mg. of pure 3α,17α,21-trihydroxypregnan-20-one. The remainder of the crystalline material (271 mg.) proved to be a mixture of unchanged substrate and tetrahydro S isomers. 3β,17α,21-Trihydroxypregnan-20-one, m.p. 224–226°, was isolated from this mixture (2.0% yield).

3α,17α,21-Trihydroxypregnan-20-one was recrystallized from acetone and finally ethanol to give small platelets, m.p. 200–204°. Various melting points were obtained with different crystallization procedures, the highest melting crystals, obtained from ethanol, melting at 214–216°, $[\alpha]^{27}D$ +60° (ethanol). The diacetate melted at 201–206°, $[\alpha]^{27}D$ +77° (ethanol). A mixture with 3α,21-diacetoxy-17α-hydroxypregnan-20-one (m.p. 195–202°) melted at 195–204°.[7]

3α-Hydroxyetiocholan-17-one by Oxidation of 3α,21-Diacetoxy-17α-hydroxypregnan-20-one with Chromic Acid.—40.5 mg. of 3α,21-diacetoxy-17α-hydroxypregnan-20-one, m.p. 201–206°, was dissolved in 4 ml. of glacial acetic acid. To this was added 20 mg. of CrO_3 in 0.4 ml. of water and the solution was allowed to stand at room temperature for 3 hours. Excess CrO_3 was reduced, the mixture diluted with water to 50 ml., and extracted with ether exhaustively to give a crude crystalline product (30.3 mg.) melting at 80–90°. Following sodium hydroxide hydrolysis at 50° for 1 hour, 11.2 mg. of 3α-hydroxyetiocholan-17-one, m.p. 140, 150–151° was recrystallized from ether–petroleum ether.

3α-Hydroxyetiocholan-17-one by Oxidation of 3α,17α,21-Trihydroxypregnan-20-one with Sodium Bismuthate.[8]—Ten mg. of 3α,17α,21-trihydroxypregnan-20-one was dissolved in 1 ml. of glacial acetic acid, followed by the addition of 1 ml. of water to a 25-ml. erlenmeyer flask covered with aluminum foil. An excess of 50 mg. of sodium bismuthate was added and the flask shaken for 0.5 hour in a Warburg at room temperature. The mixture was diluted with water, filtered and thoroughly extracted with ether. The ether solution was washed once with 1 *N* sodium hydroxide, followed by water. The dried extract consisted of white crystalline material weighing 7 mg. (70%), m.p. 140°. Recrystallization from ether–petroleum ether gave needles of 3α-hydroxyetiocholan-17-one, m.p. 140, 150–151°.

3β-Hydroxyandrostan-17-one from 3β,17α,21-Trihydroxyallopregnan-20-one by Oxidation with Sodium Bismuthate.—26.3 mg. of 3β,17α,21-trihydroxyallopregnan-20-one was dissolved in 2 ml. of glacial acetic acid followed by the addition of 2 ml. of water in a 25-ml. erlenmeyer flask covered with aluminum foil. An excess of 100 mg. of solid sodium bismuthate was added and the flask shaken for 0.5 hour in a Warburg at room temperature. The extraction with ether as previously described yielded 23 mg. (87%) of a white crystalline product, m.p. 167–170°. Recrystallization from methanol yielded 3β-hydroxyandrostan-17-one, m.p. 172–176°.

An attempt to oxidize a 21-desoxy analog, 3β,17α-dihydroxyallopregnane-20-one, m.p. 242–249°, with sodium bismuthate was unsuccessful and yielded only the starting material.

(6) All melting points were taken on a Fisher–John apparatus and are uncorrected. Infrared analyses were carried out with a Perkin-Elmer model 12-C infrared spectrometer. The analyses and interpretations were carried out by Dr. H. Rosenkrantz and Mr. P. Skogstrom in our laboratories.

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Observations on the Crystalline Forms of Galactose

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In an investigation[3] concerned with the examination of the hydrolyzate from a polysaccharide, it was necessary to recognize and identify the common crystalline forms of galactose. Five crystalline phases exist for this sugar. These are the racemate and the α- and β-pyranose forms for each enantiomorph. α-D-Galactopyranose is the stable form obtainable under the usual laboratory conditions. No record of the melting point of β-D-galactopyranose could be found. A sample of this material was prepared according to the directions of Hudson and Yanovsky.[4] It exhibited the mutarotatory properties reported by these authors and its X-ray powder diffraction diagram was that recorded by Werner[5] except that we found no evidence of contamination with the α-D-anomer. This preparation melted at 143–145°[6] when heated rapidly to near its melting point. On maintaining the temperature at 140°, the substance resolidified completely and then remelted at 164–167°. In some preparations an initial melting point at 147–150° was found and under continued slow heating, resolidification was noted at 152–155° with re-

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(6) All melting points are corrected and were observed in Pyrex glass.