Numerous investigations on the biotransformation of steroids have shown that the principal pathway for formation of cortisol and corticosterone in the adrenal gland is through progesterone (1-3); e.g. if pregnenolone is used as a corticosteroid precursor, it is first transformed into progesterone in the classical scheme of reactions. Such findings demonstrate that hydroxylations at positions 11, 17, and 21 occur in compounds with the A5-3 keto structure. It has therefore been assumed that the 3β-hydroxysteroid dehydrogenase system (4) acts before hydroxylations at 11, 17, and 21 positions; e.g. if pregnenolone is used as a corticosteroid precursor, it is first transformed into progesterone in the classical scheme of reactions. Such findings demonstrate that hydroxylations at positions 11, 17, and 21 occur in compounds with the A5-3 keto structure. It has therefore been assumed that the 3β-hydroxysteroid dehydrogenase system (4) acts before hydroxylations at positions 11, 17, and 21 positions. This is proof that the 3β-hydroxysteroid dehydrogenase system of the adrenal gland is active even after hydroxylation at the 17-position. Evidence that the 3β-hydroxysteroid dehydrogenase system of bovine adrenal glands is active in vitro after substitution of hydroxyl groups at positions 17, 21, or both on the pregnenolone molecule. Recently, Welicky and Engel (5), incubating 17α-hydroxypregnenolone-7α-H3 and progesterone-4-C14 with slices of a human adrenal tumor, found higher incorporation of H3 than of C14 into cortisol. It was then demonstrated that the 3β-hydroxysteroid dehydrogenase system of the adrenal gland is active even after hydroxylation at the 17-position.

Evidence that the 3β-hydroxysteroid dehydrogenase system of bovine adrenal glands is active in vitro after substitution of hydroxyl groups at positions 17, 21, or both on the pregnenolone molecule is presented here.

**EXPERIMENTAL PROCEDURE**

**Materials**—Steroids: 3β, 21-dihydroxy-Δ5-pregnen-20-one (Elite Chemical Company, Newark, N.J.) and 3β, 17α, 21-trihydroxy-Δ5-pregnen-20-one (Steraloids, Inc., Queens, N.Y.) were purified by paper chromatography and used as precursors. Separate solutions of these steroids in warm (37°) propylene glycol (10 mg per ml) were prepared.

Coenzyme: diphosphopyridine nucleotide (DPN, Sigma grade). A solution of DPN in phosphate buffer (12.5 μmoles per flask) was added at the time of homogenization of the glands to insure uniform distribution. The coenzyme solution was then added (12.5 μmoles of DPN per flask), and the incubation was carried out for 3 hours at 37.5° with continuous agitation. Each steroid was incubated in triplicate, and all incubations were stopped with acetone. A fourth flask in each series prepared in the same way was not incubated, 200 ml of acetone buffer. The appropriate steroid solution (0.2 ml of precursor per mg) was added immediately. These flasks are the "zero controls.

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1 In the present article, the following names are used: progesterone, Δ5-pregnen-3,20-dione; pregnenolone, 3β-hydroxy-Δ5-pregnen-20-one; 17α-hydroxypregnenolone, 3β, 17α-dihydroxy-Δ5-pregnen-20-one; deoxytocotiron, 17α, 21-trihydroxy-Δ4-pregnen-3,20-dione; cortisol, 11β, 17α, 21-trihydroxy-Δ4-pregnen-3,20-dione; corticosterone, 11β, 21-dihydroxy-Δ4-pregnen-3,20-dione.

Radioactive steroids: deoxycorticosterone-4-C14 (New England Nuclear Corporation), with specific activity of 3.3 × 106 c.p.m. per mg, and deoxycorticosterone-4-C14, with specific activity of 3.5 × 106 c.p.m. per mg were used.

**Incubation Procedure**—Each incubation flask contained 10 g of homogenized adrenal tissue suspended in 200 ml of phosphate buffer. The appropriate steroid solution (10 mg of precursor per flask) was added at the time of homogenization of the glands to ensure uniform distribution. The coenzyme solution was then added (12.5 μmoles of DPN per flask), and the incubation was carried out for 3 hours at 37.5° with continuous agitation. Each steroid was incubated in triplicate, and all incubations were stopped with acetone. A fourth flask in each series prepared in the same way was not incubated, 200 ml of acetone being added immediately. These flasks are the "zero controls.

**Extraction Procedure**—All of the flasks, including the zero controls, were extracted four times with 200 ml of warm acetone and filtered through Whatman No. 1 filter paper. The acetone was then evaporated at 40° under vacuum. Total lipids were extracted from the aqueous residue by partitioning three times with chloroform (by volume). The chloroform was completely removed at 40° under vacuum. The lipid fractions were redissolved in methanol and centrifuged at 6000 r.p.m. for 30 minutes at -20°. The supernatant methanol fractions containing the steroids and part of the lipids were decanted, and the yellow, fatty precipitates were disposed of. The methanol was evaporated under nitrogen; the steroid fractions were then chromatographed in the hexane-benzene-formamide (3β, 21-dihydroxy-Δ5-pregnen-20-one incubations) or the chloroform-formamide systems (3β, 17α, 21-trihydroxy-Δ4-pregnen-20-one incubations) as described by Zaffaroni (6).

**Identification of Steroids**—The chromatograms were viewed over an ultraviolet scanner (7), and a triphenyltetrazolium test was performed (6). Zones with a coinciding ultraviolet absorption spectrum and positive TPT2 test were eluted with methanol, and identification of the steroids was undertaken (8).

When zones from incubations of 3β, 21-dihydroxy-Δ5-pregnen-20-one were involved, purified deoxycorticosterone-4-C14 was added in trace amounts (9.0 × 104 c.p.m. = 27 μg) and then individually rechromatographed in hexane-benzene-formamide for 3 hours. The procedure followed for the identification of

*The abbreviation used is: TPT, triphenyltetrazolium.
deoxycorticosterone was as follows. The zone containing C\(^{14}\) and ultraviolet- and TPT-positive material was eluted with methanol and divided into three portions. One was analyzed as such; another was acetylated (6), and the third was oxidized (6). The three portions were then rechromatographed in the appropriate systems: both the original product and its acetylated derivative were run in hexane-benzene (1:1):formamide for 3 hours; the oxidized derivative was chromatographed in the chloroform-formamide system for 3 hours. Examination of the chromatograms in a strip counter showed that the three fractions maintained their C\(^{14}\) content. They all had absorption maxima at 240 m\(\mu\) in methanol, indicating the presence of an \(\alpha,\beta\)-unsaturated ketone in the molecule. The original compound \((R_F, 0.29)\) and its acetate \((R_F, 0.75)\) gave a positive TPT reaction, indicating the presence of an \(\alpha\)-ketol side chain. The oxidized sample \((R_F, 0.45)\) was negative to the TPT reagent but gave a blue color with potassium iodide (6) reagent, indicating that the compound had been oxidized to an etioacid. An aliquot of each fraction was used for the determination of absorption maxima in sulfuric acid (9). Their absorption maxima (2 hours) corresponded to those of deoxycorticosterone (290, 370, and 440 m\(\mu\)), deoxycorticosterone 21-acetate (290, 370, and 440 m\(\mu\)), and 3-oxo-\(\Delta^4\)-androstene-173-carboxylic acid (295 m\(\mu\)), respectively. The remainder of each portion was used for the determination of specific activities; the original, acetylated, and oxidized fractions had specific activities of 4.49, 4.95, and 4.51 c.p.m. per pmole, respectively. It was concluded that the unknown, nonradioactive compound isolated from the incubation was, in fact, deoxycorticosterone. The average conversion of 3\(\beta\),21-dihydroxy-\(\Delta^4\)-pregnen-20-one to deoxycorticosterone was 65%. Corticosterone was not detectable in these incubations. No conversion products were found in the zero controls.

B. The compound formed from incubations of 3\(\beta\),17\(a\),21-trihydroxy-\(\Delta^4\)-pregnen-20-one was analyzed as follows. The unknown ultraviolet- and TPT-positive material was eluted with pure deoxycortisol-4-C\(^{14}\) (3.3 \(\times\) 10\(^4\) c.p.m. = 95 \(\mu\)g) and rechromatographed in chloroform-formamide for 3 hours. The zone with a coinciding ultraviolet absorption spectrum, positive triphenyltetrazolium reaction, and C\(^{14}\) content was eluted with methanol and divided into three portions, which were handled in a similar manner as described previously for deoxycorticosterone.

One portion was chromatographed in chloroform-formamide for 3 hours, another portion was acetylated and chromatographed in hexane-benzene (1:1):formamide for 7 hours, and a third portion was oxidized and chromatographed in hexane-formamide for 7 hours. The original compound \((R_F, 0.60)\) and its acetylated and oxidized derivatives exhibited ultraviolet absorption maxima in methanol at 240 m\(\mu\), indicating an \(\alpha,\beta\)-unsaturated ketone in the molecule. Both the original compound and its acetate gave a positive triphenyltetrazolium reaction, characteristic of an \(\alpha\)-ketol side chain. The absorption maxima in H\(\text{H}_2\text{SO}_4\) for the original compound were found at 290 and 540 m\(\mu\) (the same as for deoxycortisol), and for the acetate, at 295 and 540 m\(\mu\) (the same as for deoxycortisol 21-acetate). Oxidation of deoxycortisol is known to yield \(\Delta^4\)-androstene-3,17-one. The oxidation product did not react with the triphenyltetrazolium reagent but gave a positive Zimmermann reaction (10), indicating a ketone at position C\(\gamma\). Its H\(\text{H}_2\text{SO}_4\) absorption maximum was at 300 m\(\mu\). Specific activities of the original compound and its acetylated and oxidized derivatives were 1.99, 1.89, and 1.81 c.p.m. per pmole, respectively.

It was concluded that the original unknown compound was deoxycortisol. The average conversion of 3\(\beta\),17\(a\),21-trihydroxy-\(\Delta^4\)-pregnen-20-one to deoxycortisol was found to be 90%. No cortisol was detected in these studies. Again, no conversion products were found in the zero controls.

**DISCUSSION**

Pregnenolone and 17\(a\)-hydroxypregnenolone have been isolated in the venous blood of adrenal glands in pigs (11) and of adrencorticotropic hormone-stimulated dogs (12). Therefore, these compounds may be considered to be normal products of the adrenal cortex. Proof exists that pregnenolone can undergo 17\(a\)-hydroxylation, resulting in the formation of 17\(a\)-hydroxypregnenolone (13).

Weliky and Engel (5) have shown that 17\(a\)-hydroxypregnenolone can be a better precursor than progesterone for the formation of cortisol. Furthermore, Lipsett and Hörff (14) demonstrated that 17\(a\)-hydroxypregnenolone-\(\Delta^4\)-3\(\beta\)-ketol will form \(\text{H}_3\)-labeled cortisol in adrenal slices from humans, guinea pigs, and rats.

Berliner et al. (15) and Cazes, Nabors, and Berliner (16) demonstrated that incubation of equimolecular quantities of pregnenolone-\(\Delta^4\)-3\(\beta\)-ketol and progesterone-4-C\(^{14}\) with bovine adrenals in a medium containing excess amounts of unlabeled C\(_3\), \(\Delta^3\)-3\(\beta\)-keto steroid intermediates resulted in an enhancement of the original \(\text{H}_3\) to C\(^{14}\) ratio in both corticosterone and cortisol. Previously Eichhorn and Hechter (17) showed that adrenocortical homogenates incubated with progesterone-C\(^{14}\) in the presence of isotonic electrolytes produced both corticosterone-C\(^{14}\) and cortisol-C\(^{14}\) with a specific activity ratio of 5 to 20:1; however, when the same homogenate was incubated with cholesterol-C\(^{14}\), the ratio was lower than 1.

Results from the present experiment demonstrate that 3\(\beta\)-hydroxysteroid dehydrogenase system activity of the bovine adrenal gland is very high, and that derivatives of 3\(\beta\)-hydroxy-\(\Delta^4\)-pregnen-20-one are readily transformed to compounds with the \(\Delta^4\)-3\(\beta\)-keto structure. Therefore, if these two compounds are formed by the adrenal gland, they would serve as excellent precursors for deoxycorticosterone and deoxycorticisol, which in turn are readily converted to corticosterone and cortisol, respectively (1).

**SUMMARY**

Incubation of homogenized bovine adrenal glands with 3\(\beta\),21-dihydroxy-\(\Delta^4\)-pregnen-20-one and 3\(\beta\),17\(a\),21-trihydroxy-\(\Delta^4\)-pregnen-20-one gave rise to deoxycorticosterone and deoxycortisol, respectively. This shows that 3\(\beta\)-hydroxysteroid dehydrogenase system is also operative on steroids with the 3\(\beta\)-hydroxy-\(\Delta^4\)-pregnen-20-one structure after hydroxylation at positions C\(_7\), C\(_{11}\), or both.

Identification of steroids was performed by paper chromatography, color reactions, spectrophotometry, derivative formation, and determination of specific activities after addition of trace amounts of known labeled steroids.

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Adrenal 3β-Hydroxysteroid Dehydrogenase Activity on C_{17}-hydroxylated Δ^5-Pregnenes, C_{21}-hydroxylated Δ^5-Pregnenes, or Both


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