Purification and Properties of a Sheep Liver 21-Hydroxysteroid Nicotinamide Adenine Dinucleotide Oxidoreductase*

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(Received for publication, July 27, 1962)

The chemical syntheses of the 21-dehydro derivatives1 of a number of C19-ketosteroids, including cortisol (3),2 cortisone (3), deoxycorticosterone (4), and Δ4-cortisone (5), have been described. The biological activities of the 11-oxygenated 21-dehydrocorticosteroids were found to be similar to the corresponding 21-hydroxysteroids. Schneider (6) reported that aqueous extracts of rat liver acetone powder reduced 21-dehydrocortisone to cortisone and suggested that the biological response to this derivative may be due to its reduction to cortisone. Since the physiological effects of other 21-dehydrosteroids may depend on their prior reduction to the corresponding 21-hydroxysteroids, we have initiated investigations of the metabolism of 21-dehydrocorticosteroids. A previous note from this laboratory (7) reported the reduction of 21-dehydrocortisol by rat tissues. This paper describes the purification and some properties of an enzyme from sheep liver that catalyzes the reversible reduction of 21-dehydrosteroids.

EXPERIMENTAL PROCEDURE

Sheep, pig, calf, and steer livers were obtained fresh from the local slaughter house. Metal-free ammonium sulfate was purchased from Mann Research Laboratories, Inc. Heavy metal ions were removed from distilled water by passage through a Bantam demineralizer or by addition of EDTA (1 mM final concentration). Alumina Cγ gel was purchased from Sigma Chemical Company. NADH and NADPH were products of Böhinger and Söhne, Mannheim, Germany, and were reported as 90% and 85.8% β form, respectively. Enzymically reduced NADPH was obtained from Mann Research Laboratories, Inc., and was reported as 82.4% pure. Various nonsteroidal carboxyl compounds were reagent grade quality and were used without further purification. Corticosteroids were purchased from Steraloids, Inc., New York. 21-Dehydrocortisol was contributed by Merek, Sharp and Dohme, Inc. and G. D. Searle and Company. This steroid aldehyde and other 21-dehydrosteroids were also synthesized in this laboratory as described below. 21-Dehydro-9α-fluorocortisol was a gift of Dr. Josef Fried. Cortisol-4-C14 (specific activity, 9.0 mc per mmole) was purchased from New England Nuclear Corporation. Radioactivity on paper chromatograms was determined with a Vanguard Autoscaner 880 flow counter (Vanguard Instrument Company) at a range setting of 3000 c.p.m. Kinetic studies were performed with a Beckman model DU spectrophotometer (Applied Physics Corporation, Monrovia, California).

Column chromatography was performed on silica gel columns, 150 x 18 mm, prepared by pouring a suspension of silica gel (Davison, 24 to 200 mesh, sifted to 100 to 200 mesh) in chloroform into the column, and washing with redistilled acetone followed by chloroform. Elution of steroids was achieved with successive use of chloroform containing from 0 to 35% acetone succinn. Steroids were also synthesized in this laboratory as described below. 21-Dehydro-9α-fluorocortisol was a gift of Dr. Josef Fried. Cortisol-4-C14 (specific activity, 9.0 mc per mmole) was purchased from New England Nuclear Corporation. Radioactivity on paper chromatograms was determined with a Vanguard Autoscaner 880 flow counter (Vanguard Instrument Company) at a range setting of 3000 c.p.m. Kinetic studies were performed with a Beckman model DU spectrophotometer (Applied Physics Corporation, Monrovia, California).

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* Aided by grants from the National Science Foundation (G-18960), The National Institutes of Health, United States Public Health Service (CY-3154 (CS)), and The American Cancer Society (P68D). The authors have previously referred to this enzyme as 21-hydroxysteroid dehydrogenase (1). At the suggestion of the editors, the enzyme has been renamed 21-hydroxysteroid NAD+ oxidoreductase, in accord with the recommendations of the International Commission on Enzyme Nomenclature (2).

1 The 21-dehydro derivatives of the 21-hydroxysteroids have been previously referred to as steroids 21-aldehydes by us and others (1, 3-7). The change in terminology, suggested by the editors, corresponds more closely to accepted steroid nomenclature, e.g. 11-dehydrosteroids.

2 The trivial names and abbreviations used are: cortisol, 4-pregnene-11β,17α,21-triol-3,20-dione; cortisone, 4-pregnene-17α,21-diol-3,20-dione; deoxycorticosterone, 4-pregnen-3α,21-pregnene-11α,21-diol-3,20-dione; deoxycorticosterone, 4-pregnen-3α,21-diol-3,20-dione; deoxycorticosterone, 4-pregnen-17α,21-diol-3,20-dione; Δ4-cortisone, 1,4-pregnadiene-1,3-21,3-17α,20-trione; Δ4-fluorocortisone, 1,4-pregnen-dien-3β,21-diol-3,20-dione; aldosterone, 3α-ol-17-one; testosterone, 4-androstene-3β,17α,20-trione; dihydrotestosterone, 4-androstene-3β,17α,20-trione; and 17α-estradiol, 3β,5α-estratriene-3,17β-diol; estrone, 1,3,5(10)-estratriene-3-ol-17-one; 21-dehydrocortisol, 4-pregnen-11β,17α-diol-3,20-dione-21-al.
Enzyoming 3 g of tissue in 12 ml of ice cold 0.25 M mixtures of 21-dehydrocortisol and cortisol, containing a total of 1 y. tetramethylammonium hydroxide and 0.5 ml of 0.2% sodium phosphate buffer, pH 6.9, in a final volume of 0.5 ml were incubated, as in Procedure 2, 4.5 ml of ethanol were added, the precipitate was discarded, and the steroids determined in a spectrophotometer. Centrifugation at 1000 × g. Livers were homogenized in a Waring Blender for 2 minutes with 4 volumes of acetonitrile previously cooled to -20°C. The acetone was removed on a Buchner funnel with suction, and the dried cake was homogenized in a second portion of chilled acetone-water. Average recoveries ranged from 20 to 30% of theory. Characteristics of the various 21-dehydrosteroids are summarized in Table I. Elemental analyses were performed by Geller Analytical Laboratories, Bardonia, New York.

RESULTS

Enzyme Purification—Preliminary survey of liver homogenates of various species indicated that sheep liver contained the highest enzymic activity as compared with steer, calf, pig, and rat livers. Sheep liver acetone powders were used for enzyme purification. Protein concentrations were measured by the method of Kalckar (16).

Livers were homogenized in a Waring Blender for 2 minutes with 4 volumes of acetonitrile previously cooled to -20°C. The acetone was removed on a Buchner funnel with suction, and the dried cake was homogenized in a second portion of chilled acetone. The cake was dried by suction on a Buchner funnel, crumbled, and allowed to dry in the air at room temperature overnight. Acetone powder, 14 g, was extracted with 140 ml of 0.1 M sodium phosphate buffer, pH 7.4, for 60 minutes at room temperature (24–27°C), cooled to 4°C, and centrifuged for 20 minutes at 1000 × g. Solid ammonium sulfate was added to the supernatant solution (100 ml) to 20% saturation (0.78 M). After 15 minutes, at 0°C, the mixture was centrifuged for 20 minutes at 1000 × g, and the precipitate was discarded.

Solid 21-Dehydrosteroids were synthesized by oxidation of the corresponding 21-hydroxysteroids with cupric acetate in methanol (5, 14). A solution of 800 μmoles of steroid alcohol and 1760 μmoles of cupric acetate were refluxed for 30 minutes in 45 ml of 95% methanol containing 3 to 4 drops of glacial acetic acid. Water, 30 ml, was added to the warm solution, and the Cu₂O was removed by filtration. Methanol was removed by distillation under reduced pressure; the steroid aldehyde was extracted with ethyl acetate and crystallized from acetone-water. Melting points of various species indicated that sheep liver contained the highest enzymic activity as compared with steer, calf, pig, and rat livers. Sheep liver acetone powders were used for enzyme purification. Protein concentrations were measured by the method of Kalckar (16).

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ammonium sulfate was added to the supernatant fluid to 50% saturation (2.0 M). The precipitate was centrifuged down and redissolved in 55 ml of water containing 1 mM EDTA at pH 7.2. The solution was brought to 55° (2 minutes), held at 55–57° for 10 minutes, rapidly cooled to 4°, and the precipitate was removed by centrifugation at 2000 x g for 10 minutes and discarded. The supernatant fluid was adjusted to pH 5.8 and alumina Cy gel (0.5 mg of solids per mg of protein) was added with stirring, which continued for 15 minutes. The suspension was centrifuged, and the supernatant fluid was set aside (see later); the precipitate was washed with 50 ml of 0.05 M sodium phosphate buffer, pH 7.4, and the washing was discarded. The gel was then extracted with 50 ml of 0.1 M sodium phosphate, pH 7.4, containing 10% ammonium sulfate. To the extract, 25 ml of saturated ammonium sulfate (4°, pH 7.5) were added, and the precipitate was centrifuged off and discarded. An additional 25 ml of saturated ammonium sulfate were added, the precipitate was collected after 15 minutes by centrifuging and was redissolved in the supernatant fluid from the original alumina Cy gel extraction (see above). The solution was adjusted to pH 5.8, and alumina Cy gel (0.5 mg of solids per mg of protein) was added, and centrifuged off after the solution was stirred for 15 minutes. The alumina was washed with 50 ml of 0.05 M sodium phosphate buffer, pH 7.4, and extracted with 50 ml of 0.1 M phosphate, pH 7.4, containing 10% ammonium sulfate. Overall purification of the enzyme starting with acetone powder ranged from 13- to 19-fold. A 19-fold purification is summarized in Table II. Enzymic activity is expressed as micromoles of NADH oxidized per minute per mg of protein. Storage at -15° for 1 year did not appreciably decrease enzymic activity.

**Stoichiometry—**21-Dehydrocortisol was stoichiometrically reduced to cortisol by the NADH-dependent oxidoreductase purified from sheep liver (Table III). The measured decrease in NADH was invariably slightly less than the changes in the other reactants. This seems to be due in part to the NAD+-dependent oxidation of the steroid solvent, propylene glycol, by a contaminating enzyme (see later). The conversion of aldehyde to alcohol was complete in 15 minutes, under the conditions used, and no further detectable changes occurred in the next 15 minutes. Increase in cortisol was equal to disappearance of 21-dehydrocortisol at each time interval studied.

**Cellular Distribution—**Although in earlier work rat liver acetone powder extracts reduced 21-dehydrocortisol equally well with NADH or NADPH (7), the purified sheep enzyme showed no activity with NADPH, indicating that the cruder extracts were capable of transhydrogenation or contained a NADPH-dependent enzyme catalyzing reduction of 21-dehydrocortisol. An examination of cell fractions (Table IV) showed that NADPH functioned as effectively as NADH in whole homogenates and in the supernatant fraction. NADH-dependent steroid aldehyde reducing activity was found predominantly in the nuclear, mitochondrial, and supernatant fractions, but NADPH-dependent activity was present to an appreciable extent only in the supernatant fraction. The sum of fractional NADH-dependent activities was far greater than the activities of the total homogenate, whereas the sum of NADPH-dependent activities approximated that of the whole homogenate. The high NADPH-dependent activity in the supernatant fraction, in contrast with the lower activity in the mitochondrial fraction, makes unlikely the possibility that this activity is due to a transhydrogenation reaction. Transhydrogenases have been found primarily in the cell particles (17, 18), although low levels have been reported in liver cell sap (19). The results indicate that there are both NADH- and NADPH-dependent enzymes present in liver capable of reducing 21-dehydrocortisol to cortisol. The properties of the NADH-requiring enzyme are described in this paper.

**pH Optima—**The pH optimum and total activity varied with the nature of the buffer. In 0.1 M sodium phosphate, the pH optimum was 6.9, whereas in 0.1 M Tris-maleate buffer, it was pH 6.2 (Fig. 1). Relative velocities at all pH values examined were lower in Tris-maleate than in phosphate buffers.

**Km Values—**Under the conditions described, velocity of reduction for the 19-fold purified enzyme was constant in the presence of excess 21-dehydrocortisol and NADH for at least 120 seconds by the spectrophotometric procedure. The Km values obtained for 21-dehydrocortisol at 30° and in 0.09 M sodium phosphate buffer, pH 6.9, ranged from 2.6 x 10^-4 to 4.6 x 10^-4 (four determinations) with a mean of 3.9 x 10^-4 M. The initial velocity of the reaction was independent of NADH concentration down to the lowest level of nucleotide (10^-4 M) and the highest level of enzyme which could be practically used in the spectrophotometric assay. Therefore, the Km with respect to NADH could not be determined directly and is probably considerably below 10^-4 M.

**Enzyme Stability—**The enzyme preparation was stable when allowed to stand at 0° for 60 minutes in phosphate buffers, pH 7.4 or 6.5, or acetate buffer, pH 5.5, and assayed at pH 6.9. Enzyme held under the same conditions at pH 4.1 (acetate buffer) or at pH 10.0 (phosphate-sodium hydroxide) lost 70 and 50% of the activity, respectively. The enzyme was stable at 55° for 1 hour and at 60° for 15 minutes at pH 6.9, but lost

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**Table II**

<table>
<thead>
<tr>
<th>Fractionation of sheep liver 21-hydroxysteroid oxidoreductase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Acetone powder extract</td>
</tr>
<tr>
<td>Ammonium sulfate precipitate (30–50%)</td>
</tr>
<tr>
<td>Supernatant fluid from heat precipitate (35°, 10 minutes)</td>
</tr>
<tr>
<td>Alumina Cy I adsorbate</td>
</tr>
<tr>
<td>Alumina Cy II adsorbate</td>
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</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>Stoichiometry of reduction of 21-dehydrocortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Component</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>21-Dehydrocortisol</td>
</tr>
<tr>
<td>Cortisol</td>
</tr>
<tr>
<td>NADH</td>
</tr>
</tbody>
</table>
Homogenates were fractionated in 0.25 M sucrose as described by Schneider and Hogeboom (13), with the isolation conditions described in Column 2. Incubation tubes contained 0.3 ml of 0.1 M sodium phosphate buffer, pH 6.9, 0.5 μmole of NADH or NADPH in 0.1 ml of phosphate buffer, pH 6.9, 1.4 μmole of 21-dehydrocortisol in 0.2 ml of 50% aqueous propylene glycol, and enzyme in 0.7 ml, final volume. Controls contained 0.2 ml of 50% aqueous propylene glycol replacing the steroid. Reaction was terminated by addition of 4.2 ml of ethanol. For zero time controls, 4.2 ml of ethanol were added before the enzyme. After centrifugation of the precipitated protein, in a final volume of 3.0 ml at 30°. Reaction was initiated after 3 minutes of prior incubation of other components by addition of enzyme. Absorbancy values were read at 15-second intervals at 340 mμ for 60 seconds; pH values were read at termination of the reaction. ————, 0.1 μ sodium phosphate buffer; ————, 0.1 M Tris-maleate buffer.

30% of its activity after 30 minutes at 60°; rapid inactivation occurred above 70°.

**Inhibitors**—Table V summarizes the effects of a number of enzyme inhibitors on the 21-hydroxysteroid oxidoreductase. Inhibition by p-mercuribenzoate, acrolein, and mercuric ions (3 × 10⁻⁴ M) indicates that sulfhydryl groups of the enzyme were involved in its activity. In no instance did the inhibition exceed 50%. At higher concentrations of mercury (10⁻³ M), the protein precipitated, resulting in complete inactivation. Neither β-mercaptoethanol nor cysteine in amounts as high as 10⁻² M overcame the inhibition by mercurobenzoate or mercuric ions, nor did these sulfhydryl compounds affect the reaction velocity in the absence of inhibitors. Complete inhibition occurred with silver ions, but other metals singly or in a mixture (including cupric and lead) at high concentrations (3 μM) had no effect. The initial reaction velocity was not influenced by addition of EDTA to the assay system.

**Table IV** 21-Hydroxysteroid oxidoreductase activity of tissue fractions with NADH and NADPH

<table>
<thead>
<tr>
<th>Tissue fraction</th>
<th>Isolation conditions</th>
<th>Experiment I</th>
<th>Experiment II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NADH μmoles/min</td>
<td>NADPH μmoles/min</td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>800 × g, 10 minutes</td>
<td>0.097</td>
<td>0.056</td>
</tr>
<tr>
<td>Nuclear</td>
<td>7,000 × g, 10 minutes</td>
<td>0.063</td>
<td>0.004</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>105,000 × g, 10 minutes</td>
<td>0.057</td>
<td>0.011</td>
</tr>
<tr>
<td>Microsomal</td>
<td></td>
<td>0.014</td>
<td>0.007</td>
</tr>
<tr>
<td>Supernatant</td>
<td></td>
<td>0.059</td>
<td>0.047</td>
</tr>
</tbody>
</table>

**Figure 1.** pH activity curves of 21-dehydrocortisol reduction. The system contained 1.1 μmoles of 21-dehydrocortisol, 0.55 μmole of NADH, 270 μmoles of buffer, and 111 μg of purified enzyme protein, in a final volume of 3.0 ml at 30°. Reaction was initiated by addition of enzyme. Absorbancy values were read at 15-second intervals at 340 mμ for 60 seconds; pH values were read at termination of the reaction. ————, 0.1 M sodium phosphate buffer; ————, 0.1 M Tris-maleate buffer.

A number of steroids were effective competitive inhibitors of 21-hydroxysteroid NAD⁺-oxidoreductase. Within the concentration range of 21-dehydrocortisol studied (0.046 to 0.37 mM), linear relationships were obtained for the reciprocal plot of reaction velocity versus 21-dehydrocortisol concentration in the presence and absence of other steroids (Fig. 2). The values obtained in order of decreasing inhibitory effectiveness (Kᵢ × 10⁻⁵ M): androsterone, 0.3; 17β-estradiol, 0.32; estrone, 0.42 (not shown in figure); testosterone, 1.4; deoxytocorticosterone, 2.2; cortisol, 2.7. Cortisol 21-acetate was not inhibitory. Dihydroxyacetone and glyoxal or methylglyoxal (each at 3 × 10⁻³ M) had no effect on the enzymic activity.

**Table V** Inhibition of 21-hydroxysteroid oxidoreductase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Mercuribenzoate (3 × 10⁻⁴ M)</td>
<td>50</td>
</tr>
<tr>
<td>Acrolein (3 × 10⁻⁴ M)</td>
<td>25</td>
</tr>
<tr>
<td>α,α-Dipyridyl (10⁻⁴ M), Dicumarol (10⁻² M), 2,4-dinitrophenol (10⁻⁴ M), sodium azide (10⁻² M), iododaceta (10⁻⁴ M), formamide (10⁻³ M), ethyl maleimide (3 × 10⁻⁴ M)</td>
<td>0</td>
</tr>
<tr>
<td>Ag⁺⁺ (3 × 10⁻³ M)</td>
<td>100</td>
</tr>
<tr>
<td>Hg⁺⁺ (3 × 10⁻⁴ M)</td>
<td>50</td>
</tr>
<tr>
<td>Al⁺⁺⁺, Cd⁺⁺⁺, Ca⁺⁺⁺, Ba⁺⁺⁺, Fe⁺⁺⁺⁺, Cu⁺⁺⁺, Pb⁺⁺⁺, Li⁺⁺, Mg⁺⁺⁺, Mn⁺⁺⁺, Zn⁺⁺⁺, Sn⁺⁺⁺⁺, Sr⁺⁺⁺⁺, Ca⁺⁺⁺, Co⁺⁺⁺, Ni⁺⁺⁺ (singly or in mixture, each at 3 × 10⁻⁵ M)</td>
<td>0</td>
</tr>
</tbody>
</table>
10^{-4} \text{M}, \text{carbonyl derivatives corresponding to the C}_{17} \text{ side chains of 21-dehydrocortisol and 21-dehydrocorticosterone, respectively, were not inhibitors. NAD}^+\text{, previously reported to inhibit the reduction by the crude rat liver preparation (7), had no effect on the purified enzyme from sheep liver.}

Substrate Specificity—Table VI shows the relative rates of reduction of a number of 21-dehydrosteroids. The initial reduction rates differed for each substrate, and did not bear any immediately evident relationship to steroid structure. The data also show that no appreciable changes in velocity ratios were observed during the purification, suggesting that a single enzyme catalyzes the reduction of the various substrates studied.

The enzyme did not reduce a variety of carbonyl-containing compounds, including acetaldehyde, glyoxal, di-glyceraldehyde, p-toluic acid, o-anisaldehyde, salicylaldehyde, and benzaldehyde. Methylglyoxal was reduced at approximately 1% of the rate of 21-dehydrocortisol. A number of steroids containing carbonyl groups in positions other than at carbon 21 were not affected by the enzyme. These included aldosterone, isandrosterone, etrone, testosteronc, cortisol, cortisone, and deoxycorticosterone. Benzaldehyde and acetaldehyde did not interfere with the reduction of 21-dehydrocortisol. Some NADH-dependent reduction of pyruvate was observed, probably owing to contamination with lactic dehydrogenase. Crystalline rabbit muscle lactic dehydrogenase did not reduce 21-dehydrocortisol. Yeast or liver alcohol dehydrogenases were also ineffective in reducing the steroid.

To ascertain whether differences in specificity existed between sheep and beef liver enzymes, reduction rates of various substrates were compared with crude acetone powder extracts. Under the conditions used, relative rates of conversion for each substrate were similar and velocities relative to 21-dehydrocortisol were comparable to those obtained with purified sheep liver enzyme (Table VII).

Reversibility—Oxidation of cortisol in the presence of NAD$^+$ could not be observed spectrophotometrically because the amount of solvent necessary to keep the required level of steroid in solution strongly inhibited the enzyme. Solvents tried were ethanol, methanol, propylene glycol, and 2-ethoxyethanol. In another approach, experiments were performed with radiocortisol. Cortisol-4-C$^{14}$ (2.8 $\mu$moles containing 670,000 c.p.m.) was incubated with 11 $\mu$moles of NADH and 1.66 mg of purified enzyme in a 3.5-ml final volume in 0.13 M phosphate buffer, pH 6.9, at 24° for 3 hours. The incubation mixture was extracted with ethyl acetate, and a portion of the extract was chromatographed on paper against authentic cortisol and 21-dehydrocortisol by the Bush B$_{4}$ system. Total counts corresponding to 21-dehydrocortisol were 360 c.p.m., and to cortisol 652,000 c.p.m. These results indicate a small degree of cortisol oxidation under these conditions.

Another experiment was performed at pH 8.5 with 13.9 $\mu$moles of cortisol-4-C$^{14}$ (containing 52,600 c.p.m.) and 2.7 $\mu$moles of NAD$^+$. After incubation as described in the preceding experiment, radioactivity was extracted into ethyl acetate. Total counts recovered were 48,000 c.p.m., or 92% of the added radioactivity. After chromatography, the radioactive area corresponding to 21-dehydrocortisol was eluted, and a portion was
TABLE VIII
Equilibrium constant of 21-hydroxysteroid oxidoreductase

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Initial concentration</th>
<th>Final concentration</th>
<th>pH</th>
<th>$K_{eq}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cortisol (F)</td>
<td>7.95 × 10^{-4}</td>
<td>6.9</td>
<td>1.02 × 10^{14}</td>
</tr>
<tr>
<td></td>
<td>NAD+</td>
<td>3.1 × 10^{-6}</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Cortisol (F)</td>
<td>3.96 × 10^{-1}</td>
<td>8.5</td>
<td>3.2 × 10^{3}</td>
</tr>
<tr>
<td></td>
<td>NAD+</td>
<td>7.75 × 10^{-4}</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Sodium phosphate buffer, 0.13 M.
- $K_{eq} = \frac{(F)(NAD^+)}{(F21A)(NADH)(H^+)}$.

![Graph](image-url)

**Fig. 3.** Elution pattern of incubation mixture after enzymic reduction of 21-dehydrocortisol. Conditions are described in text. Fractions of 7 ml were collected. O---O, absorbancy at 250 μμ of dried 1-ml aliquots redissolved in 5 ml of ethanol; •---•, absorbancy at 510 μμ after blue tetrazolium reaction. Elution patterns of cortisol and 21-dehydrocortisol were determined in runs with known compounds. Reaction of blue tetrazolium with 21-dehydrocortisol resulted in color intensity 18% that of an equal concentration of cortisol.

![Graph](image-url)

**Fig. 4.** Spectra of steroids in concentrated sulfuric acid. Spectra were run 2 hours after addition of acid to steroid. Cortisol, 40 μg; 21-dehydrocortisol 15 μg.

Isolation of Reaction Product—Cortisol formation from 21-dehydrocortisol was established as follows. 21-Dehydrocortisol, 16 mg in 4 ml of 50% propylene glycol, was mixed with 16 mg of NADH in 27 ml of 0.1 M sodium phosphate buffer, pH 7.4. After equilibration of the mixture at 37°, 4 ml (4.4 mg of protein) of the 19-fold purified oxidoreductase were added. Progress of the reaction was followed in aliquots of the incubation mixture by determining the increase in blue tetrazolium-reacting material. Reaction was complete in approximately 5 minutes. After 20 minutes, the steroids were extracted with methylene chloride. Solvent was removed by vacuum distillation, the residue was dissolved in a few milliliters of ethanol, transferred to 15-mm circles of filter paper and fractionated on a silica gel column rechromatographed with authentic 21-dehydrocortisol carrier with which it ran in an identical manner. A second portion was shown to have in sulfuric acid a spectrum identical with that of authentic 21-dehydrocortisol treated in the same way. The results of these experiments, which are summarized in Table VIII, indicate that the equilibrium constant of the enzyme is of the order of magnitude of 10^{-4}, favoring reduction. At pH values above 9, difficulties were encountered because of the rapid inactivation of enzyme, alkaline cleavage of NAD^+, and spontaneous nonenzymic reaction between cortisol and NAD^+ (20).

In the absence of steroid, there appeared to be an enzyme-catalyzed reduction of NAD^+ by the steroid solvent used. After an initial lag of approximately 60 seconds, reduction of NAD^+ at pH 6.9 occurred at approximately the following rates (absorbancy units × 10^4 per 5 minutes): propylene glycol, 2; ethylene glycol, 2; ethanol, 1; methanol, 1. When 21-dehydrocortisol was added to the system, there was an immediate, rapid decrease in absorbancy resulting from the reoxidation of NADH.
(18 × 150 mm) with the use of chloroform with increasing concentrations of acetone as eluent. The fractions were collected, concentrated, and crystallized from ethanol-cyclohexane mixtures. The elution pattern is shown in Fig. 3. The cortisol position was located by the blue tetrazolium reaction; 21-dehydrocortisol yielded little or no color with blue tetrazolium at the concentrations of steroid used. Twelve milligrams of a white material were obtained from the fractions eluted with 20 to 25% acetone in chloroform (Fractions 68 to 88). The product gave a spectrum in sulfuric acid identical with cortisol (maxima: 473, 393, 280 mp), but different from that of 21-dehydrocortisol (maxima: 460 (shoulder), 400, 325 (shoulder), and 287 mp) (Fig. 4). The melting point was identical with that of cortisol (200–212°, uncorrected, Fisher-Johns apparatus), and showed no depression when the product was mixed with cortisol. On paper chromatograms, the compound and cortisol showed identical RF values in two solvent systems (Bush B1, and formamide-benzene), and the acetylated derivative gave the same RF values as cortisol 21-acetate in the same systems.

**DISCUSSION**

An enzyme is described in this paper that catalyzes the reversible reduction of 21-dehydrosteroids to 21-hydroxysteroids.

\[
\begin{array}{c}
\text{HC=O} \\
\text{C=O} \\
\text{H} \\
\text{R'} \quad \text{R} \\
\text{O} \\
\end{array}
\text{+ NADH + H}^+ \rightleftharpoons 
\begin{array}{c}
\text{C=O} \\
\text{H} \\
\text{R'} \quad \text{R} \\
\text{O} \\
\end{array}
\text{+ NAD}^+
\]

The equilibrium of this reaction is strongly in the direction of alcohol formation. The corresponding 21-hydroxysteroids, and the C19 carbonyl of aldosterone as well as other nonsteroidal carbonyl-containing compounds were not reduced by the enzyme under the conditions studied. Since specificity of the enzyme seems to be limited to the interconversion of 21-hydroxysteroids and 21-dehydrosteroids, it is referred to as a 21-hydroxysteroid NAD⁺-oxidoreductase. The inability of the enzyme to catalyze reductions of carbonyl groups of steroids at carbon atoms 3, 11, or 20, or of the 4,5-double bond, in the presence of NADH or NADPH indicates that this enzyme is not identical with other known steroid dehydrogenases or reductases (19–23).

The evidence obtained suggests the presence of both NAD⁺- and NADP⁺-requiring 21-hydroxysteroid oxidoreductases in sheep liver. Particle-free supernatant fractions of liver homogenates reduced 21-dehydrocortisol in the presence of either NADH or NADPH. Since the absence of mitochondria made it unlikely that NADPH was reducing NAD⁺ by a transhydrogenation reaction, it was concluded that at least two separate enzymes were present in sheep liver, only one of which was purified by the procedure described.

Some biological effects of a number of 21-dehydrosteroids have been reported. Although 9α-fluorocortisol shows strong glucocorticoid activity, 21-dehydro-9α-fluorocortisol is inactive by the same criteria (24). The mineralocorticoid effect of 21-dehydro-Δ¹-cortisone is much less than that of Δ¹-cortisone, although in other respects the two compounds have similar activity (5). The 21-dehydro derivatives of cortisol and cortisone caused adrenal atrophy, thymic involution, and glycogen deposition (3). 21-Dehydrodeoxycorticosterone, on the other hand, was only one-twentieth as active as deoxycorticosterone, with the use of a work test as criterion (4). These observations suggest that the physiological effects of certain of the 21-dehydrocorticosteroids do not always parallel those of the corresponding 21-hydroxysteroids. It might seem reasonable that comparable biological activities would be observed if the effects of the 21-dehydrosteroids depended on their prior reduction in vivo by the enzyme system described in this paper. However, it is clear from the examples cited above that the effects in vivo of 21-dehydrosteroids cannot always be predicted from observations in vitro.

21-Dehydrocortisol reduction was competitively inhibited by a number of steroids. Apparently, considerable variation in the steroid nucleus is permissible, since C₁₈ and C₁₉ steroids inhibited strongly. The presence of a side chain at carbon 17 diminished the inhibitory influence. Cortisol 21-acetate was not inhibitory. These results suggest that all or part of the steroid nucleus is reversibly bound at or near the active site and that the side chain diminishes the degree of binding. The lack of effect of dihydroxycetone on the reduction indicates that this functional grouping itself is not significantly bound to the active site. Similarly, glyoxal was not reduced by the oxidoreductase, nor did it inhibit the reduction of 21-dehydrocortisol, implying that glyoxal was not appreciably bound to the enzyme. Methylglyoxal was reduced very slowly, indicating that it, too, was not a substrate. The glyoxal derivative, 21-dehydrocorticosterone, however, was readily reduced, consistent with the hypothesis that the specificity of the enzyme for 21-dehydrosteroids depends in part on the binding of the steroid nucleus to the active site on the enzyme.

It was calculated that the livers of the various species studied were capable of reducing 4 × 10⁻³ to 6 × 10⁻³ µmole of 21-dehydrocortisol per mg of whole liver per minute, an extraordinarily rapid rate considering the relatively low level of corticosteroids normally present in tissues. Neher and Wettstein (25) have reported the isolation of 11β,18-dihydroxy-3-keto-4-etiene acid from hog adrenal glands, and other workers have reported the isolation of 3-keto-4-etiene acid after perfusion of deoxycorticosterone through rat livers (26) and bovine adrenals (27). Schneider (28) observed formation of several etiene acids as a result of the incubation of guinea pig liver slices with deoxycorticosterone. Although the equilibrium constant for the 21-hydroxysteroid oxidoreductase described in this paper is strongly in favor of reduction, it may be postulated that the enzyme could participate in the synthesis of etiene acids by catalyzing the first step in the oxidation of the C₁₉ side chain of steroids.
SUMMARY

An enzyme that catalyzes the reduction of 21-dehydrosteroids to the corresponding 21-alcohols has been purified 19-fold from sheep liver acetone powders. Reduced nicotinamide adenine dinucleotide (NADH) is a cofactor requirement. Other carbonyl compounds were not reduced. The enzyme has been named 21-hydroxysteroid NAD\(^+\)-oxidoreductase. The reaction was reversible, with the equilibrium strongly favoring reduction. Reagents that react with sulfhydryl groups inhibited the reduction, and other steroids, including androgens, estrogens, and corticosteroids, were competitive inhibitors. A single enzyme appeared to reduce all the steroid aldehydes tested. Evidence is presented for the presence of a second reduced nicotinamide dinucleotide phosphate-dependent 21-hydroxysteroid oxidoreductase in sheep liver. The preparation and properties of a number of 21-dehydrosteroids are described.

Acknowledgment—We wish to acknowledge the valuable assistance of Mrs. Barbara Jacobson and Mrs. Judith Ben-Ezzer.

REFERENCES

Purification and Properties of a Sheep Liver 21-Hydroxysteroid Nicotinamide Adenine Dinucleotide Oxidoreductase
Carl Monder and Abraham White


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