20-Ketosteroids

The determination of adrenal cortical hormones in blood plasma and tissues is of clinical and biochemical importance. The method[1] used most frequently is not specific. Therefore preliminary purification of the plasma extract by chromatography, which makes the method more specific but more complicated, has been recommended[2]. In contrast, the enzymatic determination of adrenal cortical hormones[3] is both simple and specific.

A. Spectrophotometric Method

Hans Joachim Hübener

Principle

20β-Hydroxysteroid dehydrogenase (20β-dehydrogenase) catalyses the reduction of 20-ketosteroids to $20\beta_F$-alcohols[+]:

(1) $\quad C{=}O + DPNH + H^+ \rightleftharpoons HO{-}C{-}H + DPN^+$

20-ketosteroid — $20\beta_F$-hydroxysteroid

The equilibrium constant for the reaction with *Reichstein*'s substance S is 3.8×10^{-4} (pH 7.3; 25° C). The equilibrium constant with cortisone and cortisol[++] is smaller still and cannot be determined accurately because of the difficulty of estimating the reactants. Under the conditions described here, more than 99% of the added 20-ketosteroid is present as the $20\beta_F$-alcohol when the equilibrium is reached. 1 mole of DPNH is oxidized for each mole of 20-ketosteroid present.

Reagents

1. Methylene chloride *)
2. Sodium sulphate, anhydrous
3. Cyclohexane **)
4. Glycerol, redistilled †)
5. Tris-hydroxymethyl-aminomethane, tris
6. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
7. Cortisol (hydrocortisone, Δ^4-pregnene-17α,11β,21-triol-3,20-dione)
8. Ethylene-diamine-tetra-acetic acid, EDTA
   sodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$
9. Hydrochloric acid, A. R., 10% (w/v)
10. Ethanol, absolute
11. 20β-Hydroxysteroid dehydrogenase
    from *Streptomyces hydrogenans*[4,5]. For an outline of the isolation procedure, see p. 483.

[+] For information with regard to the nomenclature (β_F) see *L. F. Fieser* and *M. Fieser*: Steroide. Verlag Chemie, Weinheim/Bergstr. 1961, p. 372.

[++] Also known as hydrocortisone.

[*] *e.g.* Merck No. 6050

[**] *e.g.* Merck No. 2832

[†] *e.g.* Merck No. 4094

[1] *R. H. Silber* and *C. C. Porter*, J. biol. Chemistry *210*, 923 [1954].

[2] *A. M. Bongovanni:* Standard Methods of Clinical Chemistry. Academic Press, New York 1958, Vol. II, p. 61.

[3] *H. J. Hübener* and *F. G. Sahrholz*, Naturwissenschaften *46*, 112 [1959].

[4] *F. Lindner*, *R. Junk*, *G. Nesemann* and *J. Schmidt-Thomé*, Hoppe-Seylers Z. physiol. Chem. *313*, 117 [1958].

[5] *G. Nesemann*, *H. J. Hübener*, *R. Junk* and *J. Schmidt-Thomé*, Biochem. Z. *333*, 88 [1960].

Purity of the enzyme preparation

The enzyme should have a turnover number of at least 1 500 moles cortisone/10^5 g. protein/min. It must be practically free from other enzymes. In particular, the lactic dehydrogenase, glucose-6-phosphate dehydrogenase and malic dehydrogenase activity should not be more than 0.01% (relative to that of the 20β-dehydrogenase).

Preparation of Solutions

I. Tris buffer (0.05 M; 0.1% EDTA; pH 7.3):
Dissolve 6.05 g. tris-hydroxymethyl-aminomethane and 1 g. EDTA-$Na_2H_2 \cdot 2H_2O$ in about 900 ml. distilled water, adjust to pH 7.3 ($\pm$ 0.05) with 10% HCl using a glass electrode and dilute to 1000 ml. with distilled water.

II. Tris buffer (*ca.* 0.015 M; 0.2% EDTA; pH 8.0):
Dissolve 2 g. EDTA-$Na_2H_2 \cdot 2H_2O$ in distilled water and make up to 1000 ml. Adjust this solution to pH 8 $\pm$ 0.2 with *ca.* 18 ml. of a solution of 10 g. tris-hydroxymethyl-aminomethane in 100 ml. distilled water.

III. Reduced diphosphopyridine nucleotide (7×10^{-3} M β-DPNH):
Dissolve 5 mg. DPNH-Na_2 in 1 ml. tris buffer (solution II).

IV. Cortisol standard solution (2×10^{-4} M):
Dissolve 72.4 mg. cortisol in absolute ethanol at 45° C and make up to 10 ml. (= stock solution, 2×10^{-2} M). Just before use dilute 0.1 ml. of this solution in a 10 ml. volumetric flask to 10 ml. with tris buffer (solution II).

V. 20β-Hydroxysteroid dehydrogenase (10 mg. protein/ml.):
Dilute the enzyme solution obtained according to the procedure on page 483, 1 : 4 with tris buffer (solution II).

Stability of the solutions

The buffer solution is stable practically indefinitely. The DPNH solution should be prepared freshly each week or should be dispensed in small portions and frozen. The cortisol stock solution keeps for *ca.* 8 weeks at 0 to 4° C; the dilute cortisol solution should be prepared freshly for each series of measurements. The enzyme solution, even when diluted, is stable for several weeks at 0° C and pH *ca.* 8.

Procedure

Experimental material

Immediately after collecting blood add heparin and within half an hour obtain the plasma by centrifuging. Add a spatula tip (*ca.* 100 mg.) of EDTA-$Na_2 \cdot 2H_2O$ and store frozen until ready for the determination.

Freeze tissue (*e.g.* rat liver) in liquid air, grind while still frozen in a mortar, and add the frozen powder (refer to p. 47) to methylene chloride.

Extraction

Pipette into 60 ml. centrifuge tubes with tapered polyethylene stoppers:

Experimental	*Control*
10 ml. plasma	10 ml. tris buffer (solution II)
20 ml. methylene chloride	0.03 ml. cortisol standard solution (IV) (containing 6×10^{-9} moles cortisol)
	20 ml. methylene chloride

Seal the tubes with the stoppers and shake for 30 min. on a mechanical shaker. Centrifuge for 10 min. at 3000 g (if the phases do not separate well, freeze the samples in dry ice, thaw and centrifuge again), suck off the lower phase of methylene chloride by means of syringe with a long V2A needle. Filter the methylene chloride phase into a 60 ml. centrifuge tube and dry with Na_2SO_4 (anhydrous). Extract the aqueous residue from the first extraction with a further 20 ml. methylene chloride as described above. Evaporate the combined methylene chloride extracts to dryness at 45°C by blowing nitrogen through the solution.

Extraction into glycerol

Dissolve the dry residue from the methylene chloride extraction in

0.8 ml. methylene chloride,

transfer with a total of

3.5 ml. cyclohexane

to a 10 ml. centrifuge with a tapered polyethylene stopper, and add

1.2 ml. glycerol.

Stopper the centrifuge tubes, shake for an hour and then centrifuge for *ca.* 30 min. at about 4500 g. Suck off the upper phase quantitatively from the lower glycerol phase by means of a thin polyvinylchloride capillary attached to a water pump. Remove as much as possible of the thin emulsion between the two phases. The (lower) glycerol phase contains the 20-ketosteroids to be analysed.

Spectrophotometric measurements

Wavelength: 334 mμ; silica cuvettes, light path: 6 cm.; temperature: 25°C. Suck up the unknown sample and the control (glycerol phases, see above) with a 1 ml. graduated pipette and wipe the tip of the pipette with filter paper.

Pipette successively into the cuvette:

0.9 ml. sample or control

1.8 ml. tris buffer (solution I)

0.025 ml. DPNH solution (III).

Mix the contents of the cuvettes with a plastic rod flattened at one end until no more mixing lines can be seen. Read the optical density E_1 against air. Mix in

0.02 ml. enzyme solution (V)

and follow the decrease in optical density until the reaction stops. Read the optical density E_2 against air. $E_1 - E_2 = \Delta E$ is used for the calculations. To check the correct functioning of the assay system, mix in

0.03 ml. cortisol standard solution (IV).

The optical density must decrease again. The result of the control indicates whether the initial extraction and the extraction into glycerol have been carried out correctly.

Calculations

From the formula $\frac{\Delta E \times V}{\varepsilon \times d}$ = μmoles compound/assay mixture it follows that:

$$\frac{\Delta E \times V_{cuv.}}{6.5 \times d} \times \frac{V_{glyc.}}{v_{glyc.}} \times \frac{10^3 \times 100}{V_{plasma}} = \text{mμmoles}^{*)} \text{ cortisol/100 ml. plasma}$$

*) 1 mμmole = 10^{-3} μmoles = 10^{-9} moles

where

ΔE = $E_1 - E_2$ = optical density decrease after addition of the enzyme
$V_{cuv.}$ = total volume of the assay mixture = 2.745 ml.
$V_{glyc.}$ = total volume of the glycerol extract = 1.2 ml.
$v_{glyc.}$ = volume of the glycerol extract taken for the assay = 0.9 ml.
V_{plasma} = volume of the plasma sample (*e.g.* 10 ml.)
6.5 = extinction coefficient [cm.^{2}/μmole] of DPNH in 30% glycerol at 334 mμ
d = light path of the cuvette = 6 cm.

For the conditions chosen in this assay the formula simplifies to:

$$\frac{\Delta E \times 9.4 \times 10^3}{\text{ml. plasma}} = \text{mμmoles cortisol/100 ml. plasma.}$$

The molecular weight of cortisol is 364: therefore 1 mμmole cortisol = 10^{-3} μmoles cortisol = 0.364 μg. cortisol. The normal cortisol content of plasma is 33–69 mμmoles/100 ml.

Details for the Determination in Tissues

Extract 2 to 3 g. of tissue, frozen in liquid air, with methylene chloride for two 2 hour periods. Further treatment and the determination are as for plasma.
In the calculations the corticosteroid content is related to the g. fresh weight (progesterone is separated by partition into methylene chloride-cyclohexane);

$$\frac{\Delta E \times 940}{\text{g. fresh weight}} = \text{mμmoles adrenal cortical hormones/g. fresh weight.}$$

Normal values for rat liver: 1 to 2 mμmoles/g. fresh weight of liver.

b) Fluorimetric Method

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Principle

According to equation (1), p. 477, stoichiometric amounts of DPN are formed in the reduction of 20-ketosteroids by 20β-dehydrogenase. *Lowry et al.*[1] have described a method for the determination of DPN by means of its fluorescence in strongly alkaline solution. If after the complete enzymatic reduction of the 20-ketosteroids the residual DPNH is destroyed, the DPN formed in the reaction can be determined fluorimetrically by treatment of the reaction mixture with alkali. The amount of DPN is equivalent to the 20-ketosteroids originally present.

Reagents

As for the spectrophotometric method, p. 477 (glycerol is not required); in addition:

12. Diphosphopyridine nucleotide, DPN
    free acid; commercial preparation, see p. 1010.
13. Quinine sulphate *)
14. Hydrochloric acid, A. R., 2.2 N
15. Sulphuric acid, A. R., 0.1 N
16. Sodium hydroxide, A. R., 10 N

*) *e.g.* from Merck, Darmstadt, Germany.
[1] *O. H. Lowry, N. R. Roberts* and *J. F. Kapphahn*, J. biol. Chemistry *224*, 1047 [1957].

Preparation of Solutions

I. Tris buffer (0.05 M; pH 7.3): see p. 478.

II. Tris buffer (0.2 M; pH 7.3):
Dissolve 24.2 g. tris-hydroxymethyl-aminomethane and 1 g. ethylene-diamine-tetra-acetate in distilled water and make up to 900 ml., adjust to pH 7.3 (± 0.05) with 10% HCl (glass electrode) and dilute to 1000 ml. with distilled water.

III. Reduced diphosphopyridine nucleotide (2×10^{-3} M β-DPNH):
Dilute solution III from p. 478 3.5 times with distilled water.

IV. Cortisol standard solution (2×10^{-4} M): see p. 478.

V. 20β-Hydroxysteroid dehydrogenase (10 mg. protein/ml.): see p. 478.

VI. Diphosphopyridine nucleotide (*ca.* 1.5×10^{-4} M and 1.5×10^{-5} M β-DPN):
a) Dissolve 5 mg. DPN in distilled water and make up to 50 ml. Determine the exact DPN content according to p. 528.
b) Dilute solution a) 10 times with distilled water.

VII. Fluorescent standard (0.1 mg. quinine sulphate/1000 ml.):
Dissolve 100 mg. quinine sulphate in 0.1 N H_2SO_4 and make up to 1000 ml.; dilute 1 ml. of this solution to 1000 ml. with 0.1 N H_2SO_4.

Procedure

Extraction

See p. 478.

Extraction into cyclohexane

Dissolve the dry residue from the methylene chloride extraction of 10 ml. plasma in
0.30 ml. methylene chloride,
add
1.50 ml. cyclohexane
0.24 ml. tris buffer (solution I).

Shake for 30 min. and then centrifuge for 15 min. at 4000 g. Discard the upper phase; the lower, aqueous phase contains about 90% of the plasma corticosteroids.

Enzymatic reaction

Use 10 ml. test tubes or centrifuge tubes for the enzymatic reaction. To determine the small amount of DPN in the DPNH preparation a blank is necessary. To check the steps "Extraction" and "Extraction into cyclohexane" a cortisol control tube is required (for the composition see p. 478 under "Extraction"). Prepare simultaneously DPN standards containing 0.15 to 8 mμmoles DPN/tube.

Experimental and cortisol control:	*Blank:*
0.08 ml. sample	0.08 ml. distilled water

DPN standards:

0.01—0.05 ml. DPN standard solutions (VI a or VI b)
0.02 ml. tris buffer (solution II)
distilled water to 0.08 ml.

Pipette into all tubes:

0.01 ml. DPNH solution (III)

and in addition pipette into the blank and DPN standards:

0.01 ml. 2.2 N HCl

and into the experimental and cortisol control tubes:

0.01 ml. 20β-dehydrogenase solution (V).

Allow to stand for 30 min. at room temperature. The dehydrogenase reaction is complete after this peroid.

Pipette into the experimental and cortisol control tubes

0.01 ml. 2.2 N HCl

and into the blank and DPN standards

0.01 ml. 20β-dehydrogenase solution (V).

Mix thoroughly, pipette into all tubes

0.30 ml. 10 N NaOH,

incubate for 30 min. in a constant temperature water bath at 38°C and then add

3.00 ml. doubly distilled water.

Use these solutions for the measurements of fluorescence.

Measurements of fluorescence

Instrument: Zeiss spectrophotometer with attachment for measuring fluorescence; arrangement A. Excitation wavelength: 366 mμ line of the Hg lamp. Wavelength: fluorescent light (monochromatic with quartz monochromator) 460 mμ; slit-width: 0.2 mm.; half band-width: about 5 mμ; cuvettes: 1 cm. light path; room temperature; final volume: 3.41 ml. Measure against a cuvette containing the fluorescent standard solution (VII) set to 100 scale divisions. Read the divisions on the transmittance scale.

Standard curve

Plot the measured scale divisions for the DPN standards against the mμmoles DPN. The intensity of the fluorescent light is proportional to amount of DPN between 0.15 and 8 mμmoles per tube. With 1 mμmole DPN in 3.41 ml. of solution the intensity is 40.5 scale divisions.

Calculations

Subtract the fluorescence of the blank*) from the fluorescence (in scale divisions) of the experimental and cortisol control tubes. Read off from the standard curve the amount of DPN corresponding to this difference. Multiplication of this value by 3 gives the mμmoles 20-ketosteroids/10 ml. plasma.

Example

Cortisol control tube:

0.03 ml. 2×10^{-4} M cortisol standard solution (= 6 mμmoles cortisol) was diluted with 10 ml. doubly distilled water. Measurements of fluorescence: 72.0 scale divisions = 1.78 mμmoles 20-ketosteroids/enzymatic reaction = 5.34 mμmoles 20-ketosteroids/sample = 89% of the amount taken.

*) With DPNH "Boehringer" the blank is 25–30 scale divisions, independent of whether plasma or water is used.

Plasma:

10 ml. of plasma from a healthy subject. Measurements of fluorescence: 49.5 scale divisions = 1.22 mμ-moles 20-ketosteroids/enzymatic reaction = 0.366 mμmoles/ml. plasma. After correction for the incomplete extraction: 0.41 mμmoles 20-ketosteroids/ml. plasma.

Note

The method would be more sensitive if DPNH preparations free from α-DPN were available.

Appendix

Isolation of 20β-Hydroxysteroid Dehydrogenase [1]

Hans Joachim Hübener

The starting material is a culture of *Streptomyces hydrogenans* grown in the presence of $\Delta^{4,17(20)}$-pregnadiene-11β,21-diol-3-one [2]. The mycelium is collected by centrifuging (*ca.* 2000 g) and is then freeze-dried.

1st Day: Suspend 390 g. freeze-dried mycelium or *ca.* 2 kg. fresh weight of mycelium in 6000 ml. triethanolamine buffer (0.05 M; pH 7.6; containing 0.1% EDTA) and expose to sonic radiation *) in a continuous flow instrument (30 ml./min.; 20 kc.; 70 W/cm.^{2}; 0 to 3°C). Centrifuge at 3000 g and 0°C. Freeze the supernatant. The activity of the supernatant: 2.6 μmoles/min./ml.; volume: 4.8×10^3 ml.

2nd Day: Thaw the frozen supernatant with tap water, pour into an 8000 ml. measuring cylinder and add solid ammonium sulphate to 50% saturation over a period of 1.5 hours. Mix continuously and adjust the pH to 7.6 with 10% NH_4OH solution when necessary. When all the ammonium sulphate is added continue to stir for a further 30 min., then centrifuge (*ca.* 5000 g) and decant the supernatant together with a loose, gelatinous precipitate. Dissolve the residual solid precipitate in about 1000 ml. EDTA solution (0.1%, adjust to pH 7.6 with triethanolamine), pour into cellophane dialysis tubes and dialyse overnight against 10000 ml. EDTA solution.

3rd Day: Change the dialysing fluid twice more and dialyse for *ca.* 3 hours each time. Transfer the contents of the dialysis sacs to a 4000 ml. measuring cylinder. The activity is now 3.7 μmoles/min./ml.; volume: 1.6×10^3 ml. To the turbid solution add phosphate gel [3] with rapid stirring and keep the pH at 6 ± 0.1 by the addition of 10% acetic acid. As soon as 80% of the activity is adsorbed (test small portions), centrifuge at 3000 g and elute the gel sediment with two or three 500 ml. portions of phosphate buffer (0.05 M; pH 7.6). Precipitate the 20β-dehydrogenase from the combined eluates with ammonium sulphate **) (55% saturation). Dissolve the precipitate in 65 ml. tris buffer (0.025 M; pH 8.7) and dialyse overnight against the same buffer. Activity of the dialysed preparation: 46 μmoles/min./ml.; volume: 65 ml.

4th Day: Add the contents of the dialysis sac to a DEAE-cellulose column (inner diameter 6 cm., packed height 40 cm.) and develop the column for 24 hours with tris buffer (0.025 M; pH 8.7) (5 to 10 ml./hour).

5th to 8th Day: Change the developing buffer: develop for 24 hours with *ca.* 4000 ml. 0.05 M tris buffer (pH 8.7), which is 0.18 M with respect to NaCl. Then elute the enzyme over a period of 2 days with 0.05 M tris buffer (pH 8.7) containing 0.23 M NaCl.

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*) Ultradisintegrator, Type UD 750, Schoeller & Co., Frankfurt/M-Süd., Germany.

**) Recrystallized from 0.2% EDTA solution.

[1] The procedure described by *H. J. Hübener* and *F. G. Sahrholz*, Biochem. Z. *333*, 95 [1960], was simplified.

[2] *G. Nesemann*, *H. J. Hübener*, *R. Junk* and *J. Schmidt-Thomé*, Biochem. Z. *333*, 88 [1960].

[3] About 1000 to 2000 ml. of a phosphate gel preparation made according to *H.-U. Bergmeyer*, personal communication.

9th to about 14th Day: Precipitate the 20β-dehydrogenase from the combined active fractions (*ca.* 1500 ml.) with ammonium sulphate**) (60% saturation). Take up the sediment in *ca.* 25 ml. EDTA solution (0.1%; adjusted to pH *ca.* 8 with triethanolamine) and centrifuge to clear. Activity of the supernatant: 48.4 μmoles/min./ml.; volume: 25 ml. Crystallize the 20β-dehydrogenase by the slow addition of ammonium sulphate**) over a period of 3 to 10 days (up to 25% saturation). Centrifuge off the crystals at 5000 g. and dissolve the sediment in *ca.* 20 ml. EDTA solution and recrystallize twice more.

Physico-chemical data[4]

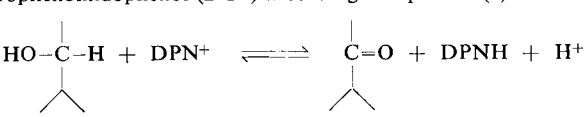
The Michaelis constant for cortisone is 5.1×10^{-5} M; for *Reichstein*'s substance S: 0.63×10^{-5} M, for cortisol: 13×10^{-5} M and for corticosterone: 24×10^{-5} M (at 25°C, pH 7.3 and 1.7×10^{-4} M DPNH). The *Michaelis* constant for DPNH is 7.2×10^{-6} M (at 25°C; pH 7.3 and 2×10^{-4} M cortisone). The turnover number is 1800 moles cortisone/min./10^5 g. protein (25°C; pH 7.3). The optimum pH is around 6.4 (25°C). The molecular weight of the enzyme is 92300 (±3%) (calculated from the sedimentation constant).

Stability of the enzyme

The enzyme is completely inactivated on heating to between 40 and 45°C. At 0°C and pH *ca.* 8, even dilute solutions are stable for several weeks in the frozen state. The enzyme can be kept as a crystalline suspension at 2 to 4°C for several years with less than a 10% loss of activity per year.

Analytical application

The enzyme can be used for the determination of 20-ketosteroids. The reverse reaction, *i.e.* the oxidation of a 20β-hydroxysteroid, occurs when the DPNH formed (equation 2) is oxidized with diaphorase and dichlorophenolindophenol (DCP) according to equation (3)[4].

(2) 

(3) $$\text{DPNH} + \text{DCP} \longrightarrow \text{DPN} + \text{reduced DCP}$$

As dichlorophenolindophenol absorbs at 600 mμ while the reduced form does not, the reaction (under anaerobic conditions) can be followed directly by spectrophotometric measurements. The method has not been tried on biological material.

Zander and *Henning*[5] have used the enzyme for the micro-analytical determination of progesterone. After extraction, purification and separation by paper chromatography, the progesterone is enzymatically reduced to Δ^4-pregnene-20β_E-ol-3-one and this is acetylated with [^{14}C] acetic anhydride. The pregnenolone acetate is chromatographed. After determination of the radioactivity in the acetate, the amount of progesterone originally present can be calculated. This method is more sensitive and more specific than any of the other known methods for the determination of progesterone.

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4) *H. J. Hübener*, unpublished. – See also *P. Talalay* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1962, Vol. V, p. 20.

5) *J. Zander* and *H. D. Henning*, personal communication.