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SYNTHESIS OF 7α- AND β-CARBOXYMETHYL DERIVATIVES OF CORTISOL, CORTICOSTERONE, DEOXYCORTICOSTERONE AND CORTISONE. IMMUNOGENIC PROPERTIES OF CORTISOL, CORTICOSTERONE AND DEOXYCORTICOSTERONE DERIVATIVES

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Summary—7α- and 7β-Carboxymethylderivatives of cortisol, corticosterone and deoxycorticosterone have been synthetized. After coupling to bovine serum albumin, they were used to elicit antibodies in rabbits. Highly specific antisera were obtained which may possibly be used for a direct radioimmunoassay of these steroids in human and rodent plasma. In the case of the derivatives of cortisol and corticosterone and stereoisomery of the coupling had an effect on the affinity and the specificity of the antisera. In all immunized rabbits the antisera obtained with the 7α-derivative had a higher affinity and a narrower specificity than the antiserum obtained with the 7β-derivative.

INTRODUCTION

Determinations of plasma levels of cortisol, corticosterone and deoxycorticosterone are employed for the diagnosis and the management of various diseases of the adrenal gland. In rodents these steroids are also assayed in various experimental studies. Moreover research in hypertension and in enzymatic defects of adrenocortical steroid biosynthesis may require the measurement of plasma deoxycorticosterone levels. A variety of methods have been described including double isotope derivative assay [1, 2], protein binding assay [1, 3, 4] and radioimmunoassay [2, 5, 6]. Many of them are non-specific or necessitate preliminary chromatographic purification of steroids.

We have previously reported a general method for the preparation of 7α- and 7β-carboxymethyl derivatives in steroid series [7–10]. This communication describes the preparation of antigens with 7α- and 7β-carboxymethyl derivatives of cortisol, corticosterone, cortisone and deoxycorticosterone, and the characteristics of the antisera obtained with cortisol, corticosterone and deoxycorticosterone antigens.

MATERIALS AND METHODS

[1,2,6,7-^{3}H]Cortisol (S.A. 80 Ci/mmol) was obtained from Amersham International (Amersham, U.K.). [1,2,6,7-^{3}H]Corticosterone (S.A. 105 Ci/mmol) and [1,2-^{3}H]Deoxycorticosterone (S.A. 46.8 Ci/mmol) were obtained from N.E.N. (Boston, U.S.A.). Their purity was checked by thin layer chromatography.

Non-radioactive steroids, chromatographically pure, were obtained from Steraloids (Wilton, U.S.A.) and Sigma (St Louis, U.S.A.). Other non-radioactive steroids were generous gifts from Roussel-Uclaf (Romainville, France).

Solvents

Carbon tetrachloride, tetrahydrofuran, diethylether, acetone, ethylacetate, methanol and diethylmalonate were of analytical grade and distilled prior to use. Deuterated dimethylsulfoxide d6 and deuterated chloroform were purchased from Centre d'Etudes Nucleaires (Saclay, France).

Physicochemical analysis

All melting points were obtained on a Leitz microscope with heating stage and have not been corrected. Infrared spectra were obtained on a Perkin–Elmer 254 (KBr pellets). Mass spectra were obtained from a NERMAG R 10.10 at 70 eV.

Nuclear magnetic resonance spectra were obtained from a Varian A 60 with tetramethylsilane as the internal standard. Microanalyses were done by the Service de microanalyse du CNRS (Thiais, France).

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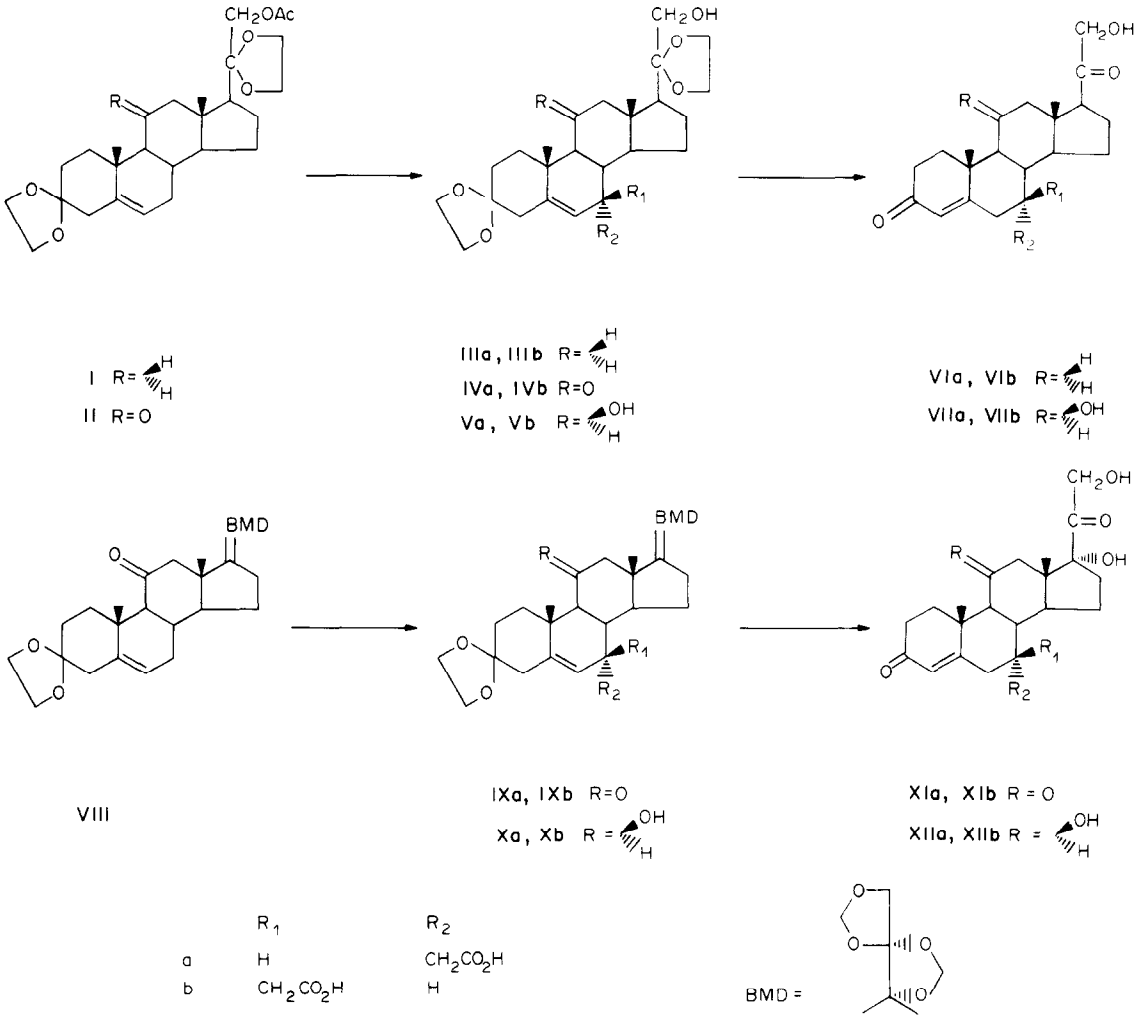

Fig. 1.

PREPARATION OF THE HAPTENS

7α- and 7β-carboxymethyl deoxycorticosterone

7α- and 7β-bromo-21-acetoxy-5-pregnene-3,20-dione 3,20 bis-*ethylene ketal.* The bromination was carried out after the functional groups had been protected [11] on the 21-acetoxy-5-pregnene-3,20-dione 3,20 *bis*-ethyleneketal (**I**) [m.p. 160–162°C]. To a solution of **I** (8.7 mmol) in CCl_4 (100 ml), anhydrous potassium acetate (8 g) was added, then the mixture was refluxed. *N*-bromo-succinimide (9.6 mmol) was rapidly added and the mixture was heated and irradiated with a 500 W photoflood lamp for 30 min. After cooling, succinimide and potassium acetate were filtered out. The CCl_4 solution was washed with $NaHCO_3$ and dried over K_2CO_3. The solution was evaporated to dryness under vacuum.

NMR (CCl_4) δ ppm: Epimer 7α: 0.78 (s, 3H, 18–CH_3), 1.02 (s, 3H, 19–CH_3), 2.0 (s, 3H, acetoxy CH_3), 3.85 (s, 4H, $-CH_2-CH_2-$ of 3-ketal), 3.91 (s, 6H, 21–CH_2 and $-CH_2-CH_2-$ of 20-ketal), 5.60 (d, J = 5 Hz, 1H, 6–CH). Epimer 7β: 0.78 (s, 3H, 18–CH_3), 1.08 (s, 3H, 19–CH_3), 2.0 (s, 3H, acetoxy CH_3), 3.83 (s, 4H, $-CH_2-CH_2-$ of 3-ketal), 3.91 (s, 6H, 21–CH_2 and $-CH_2-CH_2-$ of 20-ketal), 5.42 (s, 1H, 6–CH).

7α-Carboxymethyl-21-acetoxy-5-pregnene-3,20-dione 3,20 bis-*ethylene ketal* (***IIIa***) *and 7β-carboxymethyl-21-acetoxy-5-pregnene-3,20-dione 3,20* bis-*ethylene ketal* (***IIIb***). The condensation with diethylsodiomalonate, saponification and decarboxylation were carried out on the crude bromo product, as follows:

In an apparatus with reflux condenser and protected from moisture, NaH (0.25 mol) [50/60 weight % in paraffin oil] and anhydrous tetrahydrofuran (THF) [40 ml] were introduced. Freshly distilled ethyl malonate (0.03 mol) was slowly added, and the mixture was agitated at room temperature for 30 min. The crude bromo compound, dissolved in anhydrous THF (100 ml), was added. After 1 day of magnetic agitation at 20°C, THF was evaporated under vacuum.

The residue was dissolved in ethanol (170 ml), KOH (16 g) and water (20 ml) were added and the

mixture was shaken at 45°C for 48 h. After evaporation of ethanol, water was added (500 ml), and extracted with diethyl ether (3 × 100 ml).

The aqueous phase was acidified by HCl, then extracted with ethylacetate (4 × 150 ml). The extracts were washed with a saturated aqueous solution of NaCl until the wash-water was neutral, then dried over sodium sulfate, and evaporated under vacuum.

The residue (a mixture of the diacid and monoacid ethylic ester) was dissolved in pyridine (100 ml). After 48 h refluxing, the pyridine was evaporated, a 5% aqueous solution of KOH (300 ml) was added, and extracted with diethyl ether (3 × 100 ml).

The ether phase was washed, first with a diluted solution of HCl, then with water, dried and evaporated under vacuum. This gave 1.3 g (yield 29% from **I**) of the mixture of 7α- and 7β-ethoxy carbonyl methyl steroids (70% α and 30% β). Saponification of these esters by alcoholic KOH for 12 h at 45°C gave a mixture of the corresponding acids.

The aqueous phase was acidified with HCl, then extracted with ethyl acetate (4 × 150 ml). The fractions of the organic phase were washed with saturated aqueous solution of NaCl until the wash-water was neutral, then dried, and evaporated under vacuum: this led to the isolation of 0.70 g (yield 17% from **I**) of a mixture of 7α- and 7β-carboxymethylsteroids (**IIIa** and **IIIb**) (25% α and 75% β epimers).

The epimers were separated by chromatography of each of two acid fractions on a silica gel column (activity **II**); benzene–ether 85:15 as eluant.

(**IIIa**) M.S. 70 eV $M^{+\cdot}$ at 476. i.r. $\sqrt{}$max cm^{-1}: 3600-2500 (OH and carboxyl OH), 1730 (carboxyl C=O). NMR ($CDCl_3$) δ ppm: 0.80 (s, 3H, 18–CH_3), 1.04 (s, 3H, 19–CH_3), 3.48 (s, 2H, 21–CH_2), 3.95 (s, 4H, –CH_2–CH_2– of 3-ketal), 3.97 (s, 4H, –CH_2–CH_2– of 20-ketal), 5.48 (d, J = 5 Hz, 1H, 6–CH).

(**IIIb**) M.S. 70 eV $M^{+\cdot}$ at 476. I.R. $\sqrt{}$max cm^{-1}: 3600–2500 (OH and carboxyl OH), 1715 (carboxyl C=O). NMR ($CDCl_3$) δ ppm: 0.80 (s, 3H, 18–CH_3), 0.98 (s, 3H, 19–CH_3), 3.50 (s, 2H, 21–CH_2), 3.95 (s, 4H, –CH_2–CH_2– of 3-ketal), 3.97 (s, 4H, –CH_2–CH_2– of 20-ketal), 5.21 (s, 1H, 6–CH).

7α-Carboxymethyl-21-hydroxy-4-pregnene-3,20-dione (**VIa**). The compound **IIIa** (1.4 mmol) was dissolved in acetone (50 ml), a 4% aqueous solution of H_2SO_4 (8 ml) was added and the mixture refluxed for 30 min. After evaporation of acetone, the residue was taken up with ethylacetate (100 ml) washed with water, dried and evaporated to dryness. The residue was crystallized from acetone–hexane; several recrystallizations in acetone–hexane gave **VIa** (m.p. 181–182°C).

Anal. calcd for $C_{23}H_{32}O_5$: C 71.10, H 8.32, O 20.59; found: 70.7, 8.4, 20.8. i.r. $\sqrt{}$max cm^{-1}: 3640-2400 (OH and carboxyl OH), 1725 (carboxyl C=O), 1675 (C=O), 1640 (conjugated C=O), 1615 (C=C). NMR (DMSOd6) δ ppm: 0.62 (s, 3H, 18–CH_3), 1.17 (s, 3H, 19–CH_3), 4.04 (s, 2H, 21–CH_2), 5.57 (s, 1H, 4–CH).

7β-Carboxymethyl-21-hydroxy-4-pregnene-3,20-dione (**VIb**). The deketalization of **IIIb**, carried out as described above (reflux 30 min), gave **VIb**, after crystallization from acetone–hexane and several recrystallizations in acetone–hexane (m.p. 184–186°C).

Anal. calcd for $C_{23}H_{32}O_5$: C 71.10, H 8.32, O 20.59; found: 70.7, 8.2, 21.2. i.r. $\sqrt{}$max cm^{-1}: 3600–2500 (OH and carboxyl OH), 1720 (carboxyl C=O). NMR (DMSOd6) δ ppm: 0.60 (s, 3H, 18–CH_3), 1.14 (s, 3H, 19–CH_3), 4.03 (s, 2H, 21–CH_2), 5.59 (s, 1H, 4-CH).

7α- and 7β-carboxymethyl corticosterone

7α- and 7β-bromo-21-acetoxy-5-pregnene-3,11,20-trione 3,20-bis-*ethylene ketal.* The bromination was carried out after the functional groups had been protected [12] on the 21-acetoxy-5-pregnene-3,11,20-trione 3,20-*bis*-ethylene ketal (**II**), accordinq to the method described for the preparation of the deoxycorticosterone derivatives.

NMR (CCl_4) δ ppm: Epimer 7α: 0.75(s, 3H, 18–CH_3), 1.16 (s, 3H, 19–CH_3), 2.03 (s, 3H, acetoxy CH_3), 3.86 (s, 4H, –CH_2–CH_2– of 3-ketal), 3.91 (s, 2H, 21–CH_2), 3.98 (s, 4H, –CH_2–CH_2– of 20-ketal), 5.55 (d, J = 5 Hz, 1H, 6–CH). Epimer 7β: 0.75 (s, 3H, 18–CH_3), 1.27 (s, 3H, 19–CH_3), 2.03 (s, 3H, acetoxy CH_3), 3.86 (s, 4H, –CH_2–CH_2– of 3-ketal), 3.91 (s, 2H, 21–CH_2), 3.98 (s, –CH_2–CH_2– of 20-ketal), 5.98 (s, 1H, 6–CH).

7α-Carboxymethyl-21-hydroxy-5-pregnene-3,11,-20-trione 3,20-bis-*ethylene ketal* (**IVa**) *and 7β-carboxymethyl-21-hydroxy-5-pregnene-3,11,20-trione 3,20*-bis-*ethylene ketal* (**IVb**). The condensation with diethyl sodiomalonate, saponification and decarboxylation were carried out as described above.

Here the saponification was complete and led to the substituted malonic acids; decarboxylation of these gave 1.7 g (yield 50% from **II**) of a mixture of equal quantities of 7α- and 7β-carboxymethyl derivatives (**IVa** and **IVb**).

These epimers were methylated by diazomethane and separated by high pressure liquid chromatography on a preparative apparatus Waters LC 500 on silica column (particules size 35–75 μ; eluant used: hexane–ethylacetate–CH_2Cl_2 v/v 20:50:30; flow rate: 200 ml/mn. Retention times were: t_0 = 2.44 mn; tr of epimer α = 24.7 mn; tr of epimer β = 21.8 mn.

Methylic ester of **IVa**: M.S. 70 eV *m/e*: 504 (M^+), 473, 431, 405, 149 (100%), 99. i.r. $\sqrt{}$max. cm^{-1}: 3450 (OH). 1730 (C=O ester), 1690 (C=O), 1665 (C=C). NMR ($CDCl_3$) δ ppm: 0.73 (s, 3H, 18–CH_3), 1.21 (s, 3H, 19–CH_3), 3.41 (s, 2H, 21–CH_2), 3.66 (s, 3H, O–CH_3), 3.90 (s, 4H, –CH_2–CH_2– of 3-ketal), 4.0 (s, 4H, –CH_2–CH_2– of 20-ketal), 5.38 (d, J = 5 Hz, 1H, 6–CH).

Methylic ester of **IVb**: M.S. 70 eV *m/e*: 504 (M^+), 473, 421, 405, 149 (100%), 99. i.r. $\sqrt{}$max. cm^{-1}: 3450

(OH), 1730 (C=O ester), 1690 (C=O), 1665 (C=C). NMR ($CDCl_3$) δ ppm: 0.73 (s, 3H, 18–CH_3), 1.6 (s, 3H, 19–CH_3), 3.46 (s, 2H, 21–CH_3), 3.65 (s, 3H, O–CH_3), 3.91 (s, 4H, –CH_2 CH_2 of 3-ketal), 4.0 (s, 4H, –CH_2–CH_2– of 20-ketal), 5.14 (s, 1H, 6–CH).

The saponification of each ester gave the corresponding acids **IVa** and **IVb**.

(**IVa**) m.p. 177–180°C. (ethylacetate). i.r. $\sqrt{}$max. cm^{-1}: 3500–2500 (OH and carboxyl OH), 1725 (carboxyl C=O), 1695 (C=O), 1665 (C=C). NMR ($CDCl_3$) δ ppm: 0.75 (s, 3H, 18–CH_3), 1.23 (s, 3H, 19–CH_3), 3.41 (s, 2H, 21–CH_2), 3.95 (s, 4H, –CH_2–CH_2– of 3-ketal), 4.0 (s, 4H, –CH_2–CH_2– of 20-ketal), 5.5 (d, J = 5 Hz, 1H, 6–CH).

(**IVb**) m.p. 195–197°C. (acetone–hexane). i.r. $\sqrt{}$max. cm^{-1}: 3500–2600 (OH and carboxyl OH), 1725 (carboxyl C=O), 1700 (C=O), 1665 (C=C). NMR ($CDCl_3$) δ ppm: 0.75 (s, 3H, 18–CH_3), 1.18 (s, 3H, 19–CH_3), 3.41 (s, 2H, 21–CH_2), 3.96 (s, 4H, –CH_2–CH_2– of 3-ketal), 4.0 (s, 4H, –CH_2–CH_2– of 20-ketal), 5.16 (s, 1H, 6–CH).

7α-Carboxymethyl-11β,21-dihydroxy-5-pregnene-3,20-dione 3,20 bis-*ethylene ketal* (**Va**)

0.5 g (1 mmol) of methylic ester of **IVa** in THF (60 ml) and 1.5 g of sodium borohydride in water (7.5 ml) were allowed to react at room temperature over 5 days. The solvents were evaporated under vacuum; the residue was extracted by methane dichloride (200 ml), washed with water, dried and evaporated to dryness. This gave 0.42 g (yield 84%) of the methylic ester of **Va**.

Methylic ester of **Va**: M.S. 70 eV *m/e*: 506 (M^+), 475, 433, 415, 149, 99 (100%). i.r. $\sqrt{}$max. cm^{-1}: 3450 (OH), 1730 (C=O ester), 1665 (C=C). NMR ($CDCl_3$) δ ppm: 1.0 (s, 3H, 18–CH_3), 1.33 (s, 3H, 19–CH_3), 3.5 (s, 2H, 21–CH_2), 3.65 (s, 3H, O–CH_3), 3.95 (s, 4H, –CH_2–CH_2– of 3-ketal), 4.1 (s, 4H, –CH_2–CH_2– of 20-ketal), 5.36 (d, J = 5 Hz, 1H, 6–CH).

The above compound was saponified in ethanol–water (9:1) [100 ml] with 10 mmol of KOH at 45°C over 15 h. By neutralization and extraction **Va** was obtained (yield 85%).

(**Va**): i.r. $\sqrt{}$max. cm^{-1}: 3500–2500 (OH and carboxyl OH), 1725 (carboxyl C=O), 1665 (C=C). NMR ($DMSOd_6$) δ ppm: 0.98 (s, 3H, 18–CH_3), 1.29 (s, 3H, 19–CH_3), 3.35 (s, 2H, 21–CH_2), 3.9 (s, 4H, –CH_2–CH_2– of 3-ketal), 4.0 (s, 4H, –CH_2–CH_2– of 20-ketal), 5.38 (d, J = 5 Hz, 1H, 6–CH).

7β-Carboxymethyl-11β,21-dihydroxy-5-pregnene-3,20-dione 3,20-bis-*ethylene ketal* (**Vb**). The same procedure was applied to the methylic ester of **IVb** (reaction time 3 days) and led to the methylic ester of **Vb** (yield 80%).

Methylic ester of **Vb**: M.S. 70 eV *m/e*: 506 (M^+), 475, 433, 415, 149, 99 (100%). i.r. $\sqrt{}$max. cm^{-1}: 3450 (OH), 1730 (C=O ester), 1665 (C=C). NMR ($CDCl_3$) δ ppm: 1.01 (s, 3H, 18–CH_3), 1.23 (s, 3H, 19–CH_3), 3.5 (s, 2H, 21–CH_2), 3.65 (s, 3H, O–CH_3), 3.91 (s, 4H, –CH_2–CH_2– of 3-ketal) 4.0 (s, 4H, –CH_2–CH_2– of 20-ketal), 5.16 (d, J = 5 Hz, 1H, 6–CH).

(**Vb**): i.r. $\sqrt{}$max. cm^{-1}: 3500–2500 (OH and carboxyl OH), 1720 (carboxyl C=O), 1665 (C=C). NMR (DMSOd6) δ ppm: 0.97 (s, 3H, 18–CH_3), 1.21 (s, 3H, 19–CH_3), 3.35 (s, 2H, 21–CH_2). 3.9 (s, 4H, –CH_2–CH_2– of 3-ketal), 4.0 (s, 4H, –CH_2–CH_2 of 20-ketal), 5.16 (s, 1H, 6–CH).

7α-Carboxymethyl-11β,21-dihydroxy-4-pregnene-3,20-dione (**VIIa**). To a solution of **Va** (1 mmol) in acetone–water (99:1, v/v) [60 ml], p toluene sulfonic acid (0.1 g) was added and the mixture was shaken at room temperature over 48 h. The acetone was evaporated and the residue was taken up in water, filtered and washed with water. Recrystallization in methanol gave **VIIa** [yield 62%] (m.p. 180–182°C).

M.S. 70 eV *m/e*: 404 (M^+), 386, 373, 345, 327 (100%). i.r. $\sqrt{}$max. cm^{-1}: 3650–2500 (OH and carboxyl OH), 1730 (carboxyl C=O), 1710 (C=O), 1660 (conjugated C=O), 1660 (C=C). NMR (DMSOd6) δ ppm: 0.90 (s, 3H, 18–CH_3), 1.48 (s, 3H, 19–CH_3), 4.06 (s, 2H, 21–CH_2), 5.6 (s, 1H, 4–CH).

7β-Carboxymethyl-11β,21-dihydroxy-4-pregnene-3,20-dione (**VIIb**). Compound **Vb** was deketalised as described above. Recrystallization in methanol gave **VIIb** [yield 78%] (m.p. 228–230°C).

M.S. 70 eV *m/e*: 404 (M^+), 386, 373, 345, 327 (100%). i.r. $\sqrt{}$max. cm^{-1}: 3650–2500 (OH and carboxyl OH), 1730 (carboxyl C=O), 1710 (C=O), 1660 (conjugated C=O), 1610 (C=C). NMR (DMSOd6) δ ppm; 0.87 (s, 3H, 18–CH_3), 1.47 (s, 3H, 19–CH_3), 4.06 (s, 2H, 21–CH_2), 5.66 (s, 1H, 4 CH).

7α- and 7β-carboxymethyl cortisone

The functional groups had been protected by action on cortisone, successively of formaldehyde giving the 17α,20,20,21-*bis*-ethylene dioxy-4-pregnene-3,11,20-trione (m.p. 252–254°C) and of 2-ethylenedioxybutane giving the 17α,20,20,21-*bis*-methylenedioxy-5-pregnene-3,11,20-trione 3-ethylene ketal (**VIII**) (m.p.–189–192°C) according to [13].

7α- and 7β-bromo-17,20,20,21-bis-*methylenedioxy-5-pregnene-3,11,20-trione 3-ethylene ketal.* The bromination of **VIII** was carried out as described above.

NMR (CCl_4) δ ppm: Epimer 7α: 0.83 (s, 3H, 18–CH_3), 1.2 (s, 3H, 19–CH_3), 3.85 (s, 4H, –CH_2–CH_2– of 3-ketal), 3.9 (s, 2H, 21–CH_2), 4.97, 5.05 and 5.23 (4H, BMD), 5.57 (d, J = 5 Hz, 1H, 6–CH). Epimer 7β: 0.85 (s, 3H, 18–CH_3), 1.28 (s, 3H, 19–CH_3), 3.85 (s, 4H, –CH_2–CH_2– of 3-ketal), 3.9 (s, 2H, 21–CH_2), 4.97, 5.02 and 5.23 (4H, BMD), 5.42 (s, 1H, 6–CH).

7α-Carboxymethyl-17α,20,20,21-bis-*methylenedioxy-5-pregnene-3,11,20-trione 3-ethylene ketal* (**IXa**) *and 7β-carboxymethyl-17α,20,20,21*-bis-*methylenedioxy-5-pregnene-3,11,20-trione 3-ethylene ketal* (**IXb**). The condensation with diethyl sodiomalonate, saponificaton and decarboxylation were carried out

as described for the preparation of deoxycorticosterone derivatives. As in the case of corticosterone derivatives, the saponification was complete. After decarboxylation a mixture of equal quantities of 7α- and 7β-carboxymethyl derivatives **IXa** and **IXb** was obtained (yield 50% from **VIII**).

These epimers were separated by chromatography over a silica gel column (activity **II**); benzene–ether 88:12 as eluant, or, after methylation by diazomethane by high-pressure liquid chromatography on a preparative apparatus Waters LC 500 on a silica gel column (particules size 35–75μ); eluant used: hexane–ethylacetate–CH_2Cl_2 v/v 20:50:30; flow rate: 200 ml/mn; retention times were: $t_0 = 2.55$ mn; tr of epimer $\alpha = 21$ mn, tr of epimer $\beta = 17.25$ mn.

Methylic ester of **IXa**: i.r. $\sqrt{}$max. cm^{-1}: 1730 (C=O ester), 1705 (C=O). NMR ($CDCl_3$) δ ppm: 0.82 (s, 3H, 18–CH_3), 1.23 (s, 3H, 19–CH_3), 3.68 (s, 3H, O–CH_3), 3.93 (s, 4H, –CH_2–CH_2– of 3-ketal), 3.95 (s, 2H, 21–CH_2), 5.0, 5.05 and 5.18 (4H, BMD), 5.4 (m, 1H, 6–CH).

(**IXa**): M.S. 70 eV *m/e*: 504 (M^+), 445, 99 (100%). i.r. $\sqrt{}$max. cm^{-1}: 3650–2500 (carboxyl OH), 1735 (carboxyl C=O), 1705 (C=O). NMR ($CDCl_3$) δ ppm: 0.82 (s, 3H, 18–CH_3), 1.22 (s, 3H, 19–CH_3), 3.95 (s, 4H, –CH_2–CH_2– of 3-ketal), 3.98 (s, 2H, 21–CH_2), 5.0, 5.05 and 5.18 (4H, BMD), 5.57 (m, 1H, 6–CH).

Methylic ester of **IXb**: i.r. $\sqrt{}$max. cm^{-1}: 1730 (C=O) ester, 1700 (C=O). NMR ($CDCl_3$) δ ppm: 0.82 (s, 3H, 18–CH_3), 1.16 (s, 3H, 19–CH_3), 3.67 (s, 3H, O–CH_3), 3.93 (s, 4H, –CH_2–CH_2– of ketal), 3.95 (s, 2H, 21–CH_2), 5.0, 5.05 and 5.18 (4H, BMD), 5.18 (s, 1H, 6–CH).

(**IXb**): M.S. 70 eV *m/e*: 504 (M^+), 445, 44 (100%). i.r. $\sqrt{}$max. cm^{-1}: 3660–2500 (carboxyl OH), 1725 (carboxyl C=O), 1705 (C=O). NMR ($CDCl_3$) δ ppm: 0.84 (s, 3H, 18–CH_3), 1.18 (s, 3H, 19–CH_3), 3.93 (s, 6H, –CH_2–CH_2– of 3-ketal and 21–CH_2), 5.0, 5.05 and 5.18 (4H, BMD), 5.18 (s, 1H, 6–CH).

7α-Carboxymethyl-17α,21-dihydroxy-4-pregnene-3,11,20-trione (**XIa**). Hydrolysis of the dioxolane and the BMD of cortisone derivatives was effected by treating **IXa** with a 40% v/v aqueous solution of formic acid (10 vol) at 100°C for 50 min.

The solution was added to water (300 ml) and extracted with ethyl acetate (3 × 150 ml). The organic fractions were washed with water, dried over sodium sulfate and evaporated under reduced pressure. This led to **XIa** after several recrystallizations in ethyl acetate and acetone–hexane [Yield 20%] (m.p. 240–243°C).

Anal. calcd for $C_{23}H_{30}O_7$; C 66.00, H 7.24, O 26.76; found: 65.6, 7.7, 26.9. i.r. $\sqrt{}$max. cm^{-1}: 3640–2450 (OH and carboxyl OH), 1735 (carboxyl C=O), 1705 (C=O), 1670 (conjugated C=O), 1615 (C=C). NMR (DMSOd6) δ ppm: 0.48 (s, 3H, 18–CH_3), 1.33 (s, 3H, 19–CH_3), 4.1 and 4.15 (2H, $J_{AB} = 19$ Hz, 21–CH_2), 5.57 (s, 1H, 4 CH).

7β-Carboxymethyl-17α,21-dihydroxy-4-pregnene-3,11,20-trione (**XIb**). The same procedure was applied to **IXb**. Several recrystallizations in ethyl acetate–ether and acetone led to **XIb**. [yield 20%] (m.p. 207–210°C).

Anal. calcd for $C_{23}H_{30}O_7$: C 66.00, H 7.24, O 26.76; found: 65.7, 7.6, 26.5. i.r. $\sqrt{}$max. cm^{-1}: 3600–2500 (OH carboxyl OH), 1705 (C=O and carboxyl C=O), 1650 (conjugated C=O), 1620 (C=C). NMR (DMSOd6) δ ppm: 0.46 (s, 3H, 18–CH_3), 1.35 (s, 3H, 19–CH_3), 4.15 and 4.4 (2H, $J_{AB} = 19$ Hz, 21–CH_2), 5.6 (s, 1H, 4–CH).

7α- and 7β-carboxymethyl cortisol

7α-Carboxymethyl-11β-hydroxy-17α,20,20,21-bis-methylenedioxy-5-pregnene-3,20-dione 3-ethylene ketal (**Xa**). In a two-neck balloon flask fitted with a reflux condenser, surmounted by a calcium chloride trap, a brome ampulla and magnetic stirring, 1.8 mmol of aluminium lithium tri-tert butoxyhydrid in anhydrous ether (30 ml) and 1.7 mmol of methylic ester of **IXa** in anhydrous THF (75 ml) were successively introduced. The mixture was heated to reflux over 72 h.

The solvents were evaporated under reduced pressure, water (100 ml) and acetic acid were added to the residue which was extracted by ether (3 × 100 ml). The extracts were washed with saturated aqueous solution of sodium bicarbonate, then with water, dried and evaporated to dryness.

This led to 1.33 mmol (yield 78%) of the methylic ester of **Xa.**

i.r. $\sqrt{}$max. cm^{-1}: 3500 (OH), 1735 (C=O ester). NMR ($CDCl_3$) δ ppm: 1.08 (s, 3H, 18–CH_3), 1.32 (s, 3H, 19–CH_3), 3.67 (s, 3H, O–CH_3), 3.92 (s, 4H, –CH_2–CH_2– of 3-ketal), 3.98 (s, 2H, 21–CH_2), 5.02, 5.03 and 5.22 (4H, BMD), 5.2 (m, 1H, 6–CH).

The above compound was saponified in ethanol–water (9:1), v/v (80 ml) with 5 mmol KOH at 45°C over 15 h. By neutralization and extraction **Xa** was obtained (yield 87%).

M.S. 70 eV *m/e*: 506 (M^+), 447, 99 (100%). i.r. $\sqrt{}$max. cm^{-1}: 3650–2450 (OH and carboxyl OH), 1715 (carboxyl C=O). NMR ($CDCl_3$) δ ppm: 1.1 (s, 3H, 18–CH_3), 1.35 (s, 3H, 19–CH_3), 3.97 (s, 6H, –CH_2–CH_2– of 3-ketal and 21–CH_2), 5.05 and 5.22 (4H, BMD), 5.4 (m, 1H, 6–CH).

7β-Carboxymethyl-11β-hydroxy-17α,20,20,21-bis-methylenedioxy-5pregnene-3,20-dione 3-ethylene ketal (**Xb**). The same procedure applied to the methylic ester of **IXb** (1.47 mmol) led to 1.13 mmol (yield 77%) of the methylic ester of **Xb**.

i.r. $\sqrt{}$max. cm^{-1}: 3500 (OH), 1730 (C=O ester). NMR ($CDCl_3$) δ ppm: 1.1 (s, 3H, 18–CH_3), 1.23 (s, 3H, 19–CH_3), 3.65 (s, 3H, O–CH_3), 3.92 (s, 4H, –CH_2–CH_2– of 3-ketal), 3.97 (s, 2H, 21–CH_2), 5.02, 5.03 and 5.2 (4H, BMD), 4.13 (m, 1H, 6–CH).

Saponification of this compound led, after treatment, to **Xb** (yield 90%).

M.S. 70 eV *m/e*: 506 ($M^{+\cdot}$), 447, 44 (100%). i.r. $\sqrt{}$max. cm^{-1}: 3660–2550 (OH and carboxyl OH), *1730* (carboxyl C=O). NMR ($CDCl_3$) δ ppm: 1.11 (s, 3H, 18–CH_3), 1.25 (s, 3H, 19–CH_3), 3.93 (s, 4H, –CH_2–CH_2– of 3-ketal), 3.97 (s, 2H, 21–CH_2), 5.05 and 5.22 (4H, BMD), 5.18 (m, 1H, 6–CH).

7α-Carboxymethyl-11β,17α,21-trihydroxy-4-pregnene-3,20-dione (**XIIa**). Hydrolysis of the dioxolane and of the BMD of cortisol was effected in the same manner as for cortisone, except that the time-reaction was shorter (10 instead of 50 min). This led to **XIIa** (yield 18%); solvent of recrystallization: ethyl acetate–acetone–hexane; (m.p. 204–205°C).

Anal. calcd for $C_{23}H_{32}O_7$: C 65.69, H 7.68, O 26.23; found: 65.5. 7.9, 26.8. i.r. $\sqrt{}$max. cm^{-1}: 3620–2400 (OH and carboxyl OH), 1705 (C=O and carboxyl C=O), 1650 (conjugated C=O), 1620 (C=C). NMR (DMSOd6) δ ppm: 0.77 (s, 3H, 18–CH_3), 1.4 (s, 3H, 19–CH_3). 4.08 and 4.58 (2H, J_{AB} = 19 Hz, 21–CH_2), 5.49 (s, 1H, 4–CH).

7β-Carboxymethyl-11β,17α,21-trihydroxy-4-pregnene-3,20-dione (**XIIb**). The same treatment was applied to **Xb**, and after several recrystallizations in ethyl acetate–ether and acetone–hexane gave **XIIb** [yield 12%] (m.p. 166–170°C).

Anal. calcd for $C_{23}H_{32}O_7$: C 65.69, H 7.68, O 26.23; found: 66.1, 7.9, 26.2. i.r. $\sqrt{}$max. cm^{-1}: 3600–2500 (OH and carboxyl OH), 1715 (C=O and carboxyl C=O), 1645 (conjugated C=O), 1615 (C=C). NMR (DMSOd6) δ ppm: 0.76 (s, 3H, 18–CH_3), 1.33 (s, 3H, 19–CH_3), 4.08 and 4.48 (2H, J_{AB} = 19 Hz, 21–CH_2), 5.57 (s, 1H, 4–CH).

Prepartion of the antigens

Each hapten was condensed with BSA according to the method of Vaughan[14]. The number of steroids incorporated per mole of BSA was measured by ultra violet absorption at 248 nm. Between 18 and 22 mol of steroids per mole of BSA were generally incorporated.

Immunization of the rabbits

Three-month-old male New Zealand rabbits were used. Each animal received initially 1 mg of steroid-bovine serum albumin conjugate and 37.5 mg of BCG vaccine (Pasteur Institute, Paris, France) dissolved in 1 ml of saline and emulsified with 1 ml of Freund complete adjuvant (Calbiochem, La Jolla, U.S.A.) at several sites on the back. An intramuscular injection of *B. pertussis* (Pasteur Institute, Paris, France) was simultaneously given.

Booster injections (1 mg of steroid–bovine serum albumin conjugate emulsified in Freund incomplete adjuvant) were given at 1 month intervals. The animals were bled 10 days after each booster injection. Undiluted sera were stored at −20°C or lyophilized.

Buffer

Sodium phosphate buffer (0.05 M pH 7.40) containing 0.1% gelatin and 0.1% sodium azide was used throughout.

Steroid binding studies

The antiserum was diluted 2000 to 50,000-fold (see Tables 1, 2, 3) with buffer and incubated 12 h at 0°C with the corresponding radioactive steroid (0.1–0.2 nM). Total volume was 0.5 ml and various concentrations of competing non-radioactive steroids were added. Bound radioactivity was measured by adding 0.5 ml of Dextran coated charcoal (0.025% Dextra T 70, Pharmacia, Uppsala, Sweden 0.25% activated charcoal, Merck, Darmstadt, Germany) suspension. After mixing the suspension for 10 s the samples were centrifuged 10 min at 2000 *g* (4°C). The radioactivity of the supernatant was counted in a mixture of 5.5 g Permablend III (Packard Instruments, Downers Grove, U.S.A.) in toluene–ethanol 7:3, v/v.

RESULTS

Each of the different derivatives was used to immunize two rabbits. All the rabbits injected with cortisol and deoxycorticosterone derivatives gave satisfactory antisera. With corticosterone derivatives only two rabbits gave satisfactory antisera.

Titer and affinity for the immunogen of the antisera

Anticortisol antisera. As may be seen in Table 1 dilutions of the antiserum at which 50% of the tracer is bound varied from 1:4000, to 1:30,000. Equilibrium association constants for [^{3}H]cortisol ranged from 1.6 to 6.6 10^9 M^{-1}. Displacement of 50% bound tracer was obtained with 85–270 pg of unlabelled cortisol. All these Antibodies are thus suitable for radioimmunoassay of cortisol but those obtained with the 7α-derivative have a higher titer and affinity than those obtained with the 7β-derivative.

Anticorticosterone antisera. Characteristics of these antisera are given in Table 2. Fifty [^{3}H]corticosterone was bound at an antiserum dilution varying from 1:2000 to 1:12,000. Equilibrium association constants were 1.6 10^9 M^{-1} and 4.8 10^8 M^{-1} for the 7α- and 7β-derivatives respectively. Fifty per cent inhibition of ^{3}H-tracer binding was obtained with 230 pg (7α-derivative) and 300 pg (7β-derivative) of unlabeled corticosterone. These antibodies are suitable for radioimmunoassay of corticosterone but as in the case of cortisol the antisera obtained with the 7α- derivative have more favorable characteristics than those obtained with the 7β-derivative.

Antideoxycorticosterone antisera

Quantitative characteristics of these antisera are shown in Table 3. Antiserum dilution at which 50% of [^{3}H]deoxycorticosterone is bound vary from 1:15,000 to 1:50,000. Equilibrium association constants ranged from 6.1 10^9 M^{-1} to 1.3 10^{10} M^{-1} and

displacement of 50% [^{3}H]deoxycorticosterone binding was obtained with 35–60 pg of unlabelled deoxycorticosterone. Thus all these antisera are suitable for radioimmunoassay of deoxycorticosterone. No clear-cut difference was observed when using 7α- or 7β-derivatives.

Steroid specificity

Anticortisol antisera. The percent cross-reactions are shown in Table 4. The main cross-reacting steroids naturally occurring in plasma are deoxycortisol and corticosterone and among synthetic glu-

Table 1. Characteristics of the anticortisol antisera

	Anti 7α-derivative		Anti 7β-derivative	
	Rabbit 85	Rabbit 86	Rabbit 87	Rabbit 88
Antiserum dilution at which 50% of ^{3}H-tracer is bound	1:30,000	1:20,000	1:6000	1:4000
Equilibrium association constant with [^{3}H]cortisol*	$6.6\ 10^9\ M^{-1}$	$6.4\ 10^9\ M^{-1}$	$1.6\ 10^9\ M^{-1}$	$2.1\ 10^9\ M^{-1}$
Mass of unlabelled cortisol inhibiting 50% of ^{3}H-tracer binding	86 pg	110 pg	270 pg	160 pg

*Obtained from Scatchard plot [36].

Table 2. Characteristics of the anticorticosterone antisera

	Anti 7α-derivative	Anti 7β-derivative
	Rabbit 18	Rabbit 19
Antiserum dilution at which 50% of ^{3}H-tracer is bound	1:12,000	1:2000
Equilibrium constant association with [^{3}H]corticosterone*	$1.6\ 10^9\ M^{-1}$	$4.8\ 10^8\ M^{-1}$
Mass of unlabelled corticosterone inhibiting 50% of ^{3}H-tracer binding	230 pg	300 pg

*Obtained from Scatchard plots [36].

Table 3. Characteristics of the antideoxycorticosterone antisera

	Anti 7α-derivative		Anti 7β-derivative	
	Rabbit 02	Rabbit 03	Rabbit 04	Rabbit 776
Antiserum dilution at which 50% of ^{3}H-tracer is bound	1:15,000	1:50,000	1:25,000	1:20,000
Equilibrium association constant with [^{3}H]deoxycorticosterone*	$6.1\ 10^9\ M^{-1}$	$3.3\ 10^{10}\ M^{-1}$	$8.1\ 10^9\ M^{-1}$	$1.3\ 10^{10}\ M^{-1}$
Mass of unlabelled deoxycorticosterone inhibiting 50% of ^{3}H-tracer binding	56 pg	34 pg	54 pg	60 pg

*Obtained from Scatchard plots [36].

Table 4. Steroid specificity of the anticortisol antisera

	Anti 7α-derivative		Anti 7β-derivative	
Steroid	Rabbit 85	Rabbit 86	Rabbit 87	Rabbit 88
Cortisol	100	100	100	100
11-Deoxycortisol	7.6–7.4*	8.3	7.5	13.4
Deoxycorticosterone	0.65	0.60	2.2	
Corticosterone	3.0–5.2*	5.8–5.5*	36	5.7
Cortisone	0.7		66.1	
17-OH-Progesterone	<0.05	0.4–0.4*	5.6	0.4
17-OH-Pregnenolone	<0.05		<0.3	
Pregnenolone	<0.05		<0.3	
Progesterone	<0.05	<0.2	2.8	
20-OH-Progesterone	<0.05	<0.2	0.6	
5α-Dihydroprogesterone	<0.05		0.45	
5β-Dihydroprogesterone	<0.05		0.40	
6β-OH-Cortisol	1.7	4.2	7.1	7.3
Prednisone	0.1		2.2	
Prednisolone	4.5–6.5*	7.3	84	47
6-Methyl prednisolone	4.5		15	
Dexamethasone	<0.05	<0.2	<0.3	
Testosterone	<0.05		<0.3	
Estradiol	<0.05		<0.3	
Aldosterone	<0.05		<0.3	

The concentration of unlabelled steroid giving a 50% inhibition of [^{3}H]cortisol binding was compared to that of unlabelled cortisol (= 100).
*Measurements performed on two different bleedings from the same animal.

Table 5. Previously described anticortisol antisera

Position of the linkage	3 oxime	3 CMO	3 CMO	3 CMO	3 CMO	3 CMO	3 CMO	3 CMO*
Reference	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[25]
Steroid abbreviation								
Cortisol	100	100	100	100	100	100	100	100
11-Deoxycortisol	0.08	58	7.1	3.8	14	10	3.98	13.0
Deoxycorticosterone	<0.01	2.8	0.2	0.06	0.3	1.6	NT	0.15
Corticosterone	0.4	23.4	4.0	12.5	1.7	10	0.99	3.5
Cortisone	0.01	54	0.7	6.6	0.3	0.2	<0.01	9.0
17-OH-Progesterone	<0.01	25	<0.01	0.06	1.4	0.8	0.96	0.1
17-OH-Pregnelone	NT	NT	NT	NT	0.04	NT	NT	NT
Pregnelone	NT	NT	NT	NT	NT	NT	NT	<0.01
Progesterone	0.8	3.2	0.01	0.001	0.2	1	<0.01	<0.01
20-OH-Progesterone	NT	NT	NT	NT	0.01	NT	NT	NT
5α-Dihydroprogesterone	NT	NT	NT	NT	NT	NT	NT	NT
5β-Dihydroprogesterone	NT	NT	NT	NT	NT	NT	NT	NT
6β-OH-Cortisol	NT	1.4	NT	0.05	NT	NT	NT	NT
Prednisone	<0.001	NT	NT	0.7	1.7	0.01	NT	NT
Prednisolone	<0.01	NT	64	28.5	26	100	NT	NT
6-Methyl prednisolone	NT	NT	NT	NT	NT	NT	NT	NT
Dexamethasone	0.01	NT	0.05	0.01	NT	1	NT	NT
Testosterone	<0.01	NT	<0.01	0.001	0.01	0.1	1.6	NT
Estradiol	<0.001	NT	<0.01	0.001	0.01	0.001	<0.01	NT
Aldosterone	<0.01	NT	<0.01	0.001	NT	0.001	NT	<0.01

Position of the linkage	21 HS	21 HS	21 HS	21 HS	21 HS	21 HS	6α CM	6β CM	7α CM	7β CM
Reference	[6]	[22]	[23]	[21]	[16]	[24]	[16]	[16]	This work	
Steroid abbreviation										
Cortisol	100	100	100	100	100	100	100	100	100	100
11-Deoxycortisol	100	18.7	NT	15.0	68	28.2	26	53	7.5	7.5
Deoxycorticosterone	46	17.5	0	3.30	8.4	4.3	1.4	4.1	0.6	2.2
Corticosterone	46	13.5	1.4	5.60	27.8	9.8	11.6	37	3.0	36
Cortisone	40	23.2	NT	8.80	65	23	1.1	58	0.7	6.1
17-OH-Progesterone	56	5.3	NT	14.0	34	4.54	10	18	<0.05	0.4
17-OH-Pregnenolone	<0.01	NT	NT	<0.01	NT	NT	NT	NT	<0.05	<0.3
Pregnenolone	NT	NT	NT	<0.01	NT	NT	NT	NT	<0.05	<0.3
Progesterone	28	2.7	5.5	2.5	12.3	14.8	2.8	5.9	<0.05	2.8
20-OH-Progesterone	NT	NT	NT	2.0	NT	NT	NT	NT	<0.05	0.6
5α-Dihydroprogesterone	NT	NT	NT	NT	NT	NT	NT	NT	<0.05	0.45
5β-Dihydroprogesterone	NT	NT	NT	NT	NT	NT	NT	NT	<0.05	0.4
6β-OH-Cortisol	NT	NT	4.0	<0.01	0.2	NT	7	5.3	1.7	7.1
Prednisone	16	11.8	NT	NT	NT	NT	NT	NT	0.1	2.2
Prednisolone	NT	36.6	NT	NT	NT	NT	NT	NT	4.5	47
6-Methyl prednisolone	NT	NT	NT	NT	NT	NT	NT	NT	4.5	15
Dexamethasone	2.0	<1.0	NT	NT	NT	NT	NT	NT	<0.05	<0.3
Testosterone	13	<1.0	NT	<0.01	NT	1.1	NT	NT	<0.05	<0.3
Estradiol	<0.01	<1.0	NT	<0.01	NT	NT	NT	NT	<0.05	<0.3
Aldosterone	<0.1	NT	0.86	NT	NT	0.93	NT	NT	<0.05	<0.3

*Cortisol 21-acetate 3 CMO BSA.
NT = Not tested, CMO = carboxymethyloxime, HS = hemisuccinate, CM = carboxymethyl.

Table 6. Steroid specificity of the anticorticosterone antisera

		Anti 7α-derivative	Anti 7β-derivative
Steroid	Rabbit	18	19
Corticosterone		100	100
Cortisol		3.1	0.22
Deoxycorticosterone		3.3	2.5
Progesterone		0.3	0.03
Aldosterone		0.21	0.27
Deoxycortisol		<0.05	<0.03
Cortisone		0.27	<0.03
17-Hydroxyprogesterone		<0.05	<0.03
17-Hydroxypregnenolone		<0.05	<0.03
20-Hydroxyprogesterone		<0.05	<0.03
18-Hydroxycorticosterone		<0.05	<0.03
Pregnenolone		<0.05	<0.03
5α-Dihydroprogesterone		<0.05	<0.03
5β-Dihydroprogesterone		<0.05	<0.03
Dexamethasone		<0.05	<0.03
Testosterone		<0.05	<0.03
Estradiol		<0.05	<0.03
18-Hydroxydeoxycorticosterone		<0.05	<0.03

The concentration of unlabelled steroid giving a 50% inhibition of [^{3}H]corticosterone binding was compared to that of unlabelled corticosterone (= 100).

Table 7. Previously described anticorticosterone antisera

Position of linkage		21 HS[a]	21 HS	21 HS	21 HS	21 HS	21 HS	21 HS	3 CMO	3 CMO	7α CM	7β CM
	Reference	[26]	[27]	[5]	[28]	[29]	[30]	[24]	[31]	[32]	This work	
Steroid abbreviation												
Corticosterone		100	100	100	100	100	100	100	100	100	100	100
Cortisol		100	0.5	17	2.8	6.9	3.6	1.7	5.9	0.027	3.1	0.18
11-Deoxycortisol		3.0	NT	3	0.8	<5	1.7	1.8	7.7	0.0016	<0.05	<0.03
Cortisone		2.5	0.003	<0.01	0.2	NT	NT	0.2	40.8	NT	0.27	<0.03
Deoxycorticosterone		4.5	4.7	33	26.1	27.9	18	32.7	100	0.03	3.3	2.5
17-OH-Progesterone		1.7	NT	59	0.2	<5	<0.1	2.0	14.8	0.006	<0.05	<0.03
17-OH-Pregnenolone		NT	NT	<0.01	NT	NT	<0.1	NT	NT	NT	<0.05	<0.03
Pregnenolone		<0.01	NT	NT	NT	<5	NT	1.4	NT	0.003	<0.05	<0.03
Progesterone		4.5	3.5	48	44.3	37.6	16	38	100	0.024	0.3	0.03
20-OH-Progesterone		2.5	NT	NT	NT	NT	NT	NT	NT	NT	<0.05	<0.03
5α-Dihydroprogesterone		NT	NT	NT	NT	NT	NT	NT	NT	NT	<0.05	<0.03
5β-Dihydroprogesterone		NT	NT	NT	NT	NT	NT	NT	NT	NT	<0.05	<0.03
Dexamethasone		NT	NT	<0.01	NT	NT	NT	NT	NT	NT	<0.05	<0.03
Testosterone		3.1	NT	9	10	<5	<0.1	2.6	4.3	0.0025	<0.05	<0.03
Estradiol		NT	NT	<0.01	<0.001	<5	<0.1	NT	<0.01	<0.001	<0.05	<0.03
Aldosterone		3.0	0.3	<0.01	NT	<5	NT	1.6	<0.1	0.001	0.21	0.27
18-OH-Corticosterone		NT	0.02	NT	NT	NT	NT	5.8	NT	0.002	<0.05	<0.03
18-OH-Deoxycorticosterone		NT	0.005	NT	NT	NT	NT	1.2	NT	0.0002	<0.05	<0.03

(a) 21-HS-corticol; NT = not tested; HS = hemisuccinate; CMO = carboxymethyloxime; CM = carboxymethyl.

cocorticoids prednisolone and methylprednisolone. 6β-Hydroxycortisol does not cross-react significantly. Cross-reactions of the antiserum obtained with 7α-derivative were lower than those of the antisera obtained with 7β-derivative. This difference in specificity is evident for corticosterone and prednisolone. A comparison with previously described anticortisol antisera is shown in Table 5. The percent cross-reactions with deoxycortisol and corticosterone were lower when the antisera were raised with the 7α-derivative than when antisera were raised with the 3,6α- or 6β-, 21-derivatives of cortisol.

Anticorticosterone antisera

The percent cross-reactons are given in Table 6. The mainly cross-reacting steroid is deoxycorticosterone but this cross-reaction is low (3%). Compared to previously described anticorticosterone antisera (Table 7) antisera raised with antigens coupled to position 7 exhibit lower cross-reactions with deoxycorticosterone, progesterone and 17-hydroxyprogesterone. No clearcut difference was observed between 7α- and 7β-derivatives.

Antideoxycorticosterone antisera

The percent cross-reactions are shown in Table 8. None of the steroids tested cross-react significantly. Antisera raised with antigens coupled to position 7 exhibit lower cross-reactions with corticosterone and progesterone than previously described antisera (Table 9).

DISCUSSION

As shown in a previous work [10] the use of antigens coupled to position 7 for raising antibodies against corticosteroids yields antisera suitable for radioimmunoassay. The stereoisomery of the coupling seems to have an effect on the affinity of the antisera obtained with cortisol and corticosterone antigens. With the 7α-derivatives the antisera have an affinity 3-fold higher (1.6 10 M^{-1} vs 6.6 10^9 M^{-1},

Table 8. Steroid specificity of the antideoxycorticosterone antisera

Steroid (abbreviation)	Anti 7α-derivative Rabbit 03	Anti 7β-derivative Rabbit 776
Deoxycorticosterone	100	100
Corticosterone	0.3	0.3
Progesterone	0.3	3.7
5α-Dihydroprogesterone	0.07	0.5
5β-Dihydroprogesterone	<0.05	0.1
11-Deoxycortisol	<0.05	0.2
Cortisol	<0.05	<0.05
Cortisone	<0.05	<0.05
17-OH-Progesterone	<0.05	<0.05
17-OH-Pregnenolone	<0.05	<0.05
Pregnenolone	<0.05	<0.05
Dexamethasone	<0.05	<0.05
Testosterone	<0.05	<0.05
Estradiol	<0.05	<0.05
Aldosterone	<0.05	<0.05
18-Hydroxycorticosterone	<0.05	<0.05
18-Hydroxydeoxycorticosterone	<0.05	<0.05

The concentration of unlabelled steroid giving a 50% inhibition of [^{3}H]deoxycorticosterone binding was compared to that of unlabelled deoxycorticosterone (= 100).

Table 9. Previously described antideoxycorticosterone antisera

Position of linkage / Reference	21 HS [31]	21 HS [30]	21 HS [33]	3 CMO [34]	3 CMO [35]	7α CM This work	7β CM
Steroid abbreviation							
Deoxycorticosterone	100	100	100	100	100	100	100
Corticosterone	3.4	1.4	3.6	0.9	0.17	0.3	0.3
Progesterone	54.3	160	100	1.8	0.63	0.3	3.7
5α-Dihydroprogesterone	NT	NT	NT	NT	NT	0.07	0.05
5α-Dihydroprogesterone	NT	NT	NT	NT	NT	<0.05	0.1
11-Deoxycortisol	0.8	5.6	90.0	0.2	0.15	<0.05	0.2
Cortisol	<0.1	0.2	NT	<0.01	0.01	<0.05	<0.05
Cortisone	<0.1	NT	NT	<0.01	NT	<0.05	<0.05
17-OH-Progesterone	1.9	<0.1	9.0	<0.01	0.03	<0.05	<0.05
17-OH-Pregnenolone	NT	<0.1	<0.1	<0.01	0.01	<0.05	<0.05
Pregnenolone	NT	NT	<0.1	<0.01	0.08	<0.05	<0.05
Dexamethasone	NT	NT	NT	NT	NT	<0.05	<0.05
Testosterone	8.3	<0.1	1.2	<0.1	0.17	<0.05	<0.05
Estradiol	<0.01	<0.1	<0.1	<0.01	NT	<0.05	<0.05
Aldosterone	0.27	NT	NT	<0.01	0.02	<0.05	<0.05
18-OH-Corticosterone	NT	NT	NT	NT	0.01	<0.05	<0.05
18-OH-Deoxycorticosterone	NT	NT	NT	NT	0.01	<0.05	<0.05

NT = Not tested. CM = carboxymethyl, CMO = carboxymethyloxime. HS = hemisuccinate.

4.8 $10^8\,M^{-1}$ vs 1.6 $10^9\,M^{-1}$ respectively). The stereoisomery of the coupling of cortisol seems also to have an effect on antibody specificity. Antisera obtained with the 7α-derivatives of cortisol exhibit 10- to 50-fold lower cross-reactions specially with progesterone and 17 OH progesterone (<0.05% vs 2.8%, <0.05% vs 5.6% respectively). These differences have not been found when analysing antiserum obtained with 7α- and 7β-derivatives of corticosterone and deoxycorticosterone. It should also be stressed that it may be difficult to generalize results obtained in such experiments since characteristics of the antisera may also depend on differences in the immunological responses of individual rabbits.

Coupling to position 7 yields antisera which compare favorably with the most specific antisera already described, specially for corticosterone and deoxycorticosterone allowing probably direct radioimmunoassay of these steroids without chromatography. This would be of great interest in pediatric and neonatal endocrinology since evaluation of adrenocorticol functions should necessitate only small volumes of plasma. For the same reasons antisera raised with antigens coupled to position 7 would be useful for the direct measurement of cortisol and corticosterone in small samples of plasma drawn from laboratory animals.

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