1α-HYDROXYCORTICOSTERONE: SYNTHESIS IN VITRO AND PROPERTIES OF AN INTERRENAL STEROID IN THE BLOOD OF CARTILAGINOUS FISH (GENUS RAJA)

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ABSTRACT

Chromatograms prepared from interrenal incubates or blood from two species of cartilaginous fish consistently failed to show the presence of cortisol, corticosterone, or aldosterone but did reveal a substance, more polar than cortisol, which gave reactions expected of a corticoid.

The substance was identified as the previously unknown 1α,11β,21-trihydroxypregn-4-ene-3,20-dione. Dehydration converted the steroid to the known 11β,21-dihydroxypregna-1,4-diene-3,20-dione. The NMR spectra confirmed the location of the hydroxy group and established the α-(axial) configuration. The steroid crystallized in needles from acetone-hexane, m.p. 201-202°, $\lambda_{max}^{methanol}$ 242.5 mμ, ε 15,600. It formed a diacetate, m.p. 182-183°. The sulphuric acid chromogen and IR spectra of the new steroid and its 1-dehydro derivative are presented.

1α,11β,21-Trihydroxypregn-4-ene-3,20-dione was produced in interrenal incubates from endogenous precursors (e.g. 6.4 μg/100 mg/hr with added ACTH) and from [4-^{14}C] corticosterone. Other products were formed only in trace amounts. From 1150 ml of blood plasma 18 μg of the steroid were isolated.

A few characteristics of the 1α-hydroxylating enzyme system are briefly discussed.

INTRODUCTION

The isolation of 1α-hydroxycorticosterone (1) from the blood and incubates of the interrenal gland of two species of cartilaginous fish (Raja) was reported in a preliminary communication (2). 1-Hydroxylated

derivatives of C_{21}-steroids are relatively rare (3-5) and where the configuration has been determined, microbiological transformation products of C_{21}-substrates have been the 1β-isomers (4,5). In many cases, the C-1 hydroxylation of a steroid by microorganisms has been substrate-specific (6), and no 1-hydroxylated derivative of corticosterone has been reported. A few plant glycoside sapogenins contain a 1β-hydroxy group (7). Although 1-hydroxylation has not previously been associated with animal tissues, Schneider and Bhacca (8) recently found 1β-hydroxylation of 3α,17α,20β,21-tetrahydroxy-5β-pregnan-11-one to occur *in vivo* in man and *in vitro* in liver slices of the guinea pig.

This paper describes the biosynthesis, identification, and some properties of 1α-hydroxycorticosterone.

MATERIALS AND METHODS

Two species of skate, *Raja radiata* Donovan and *Raja ocellata* Mitchill caught off the Atlantic coast of Canada, were held alive in tanks of recirculating refrigerated sea water. The animals were anaesthetized with tricaine methanesulphonate at a dilution of 1:3000 in sea water. Blood was collected from the heart by cannulation of the conus arteriosus and the plasma separated immediately by centrifugation in the cold. Heparin was used as an anti-coagulant. The interrenal gland, an encapsulated organ of ca. 100-600 mg in adult animals, was exposed dorsally, removed, cleaned of foreign tissue, and held in a small volume of ice-cold incubation medium under oxygen until sufficient glandular tissue was collected for an incubation.

Incubations of Interrenals

Incubations were done in a water-bath shaker at 26°C in an oxygen atmosphere. A medium developed by Bern and Nandi (9) for use with teleostean tissues was modified to increase its buffering capacity. The composition of the medium in g/l was as follows: NaCl, 11.2; KCl, 0.373; $CaCl_2$, 0.333; $MgCl_2.6H_2O$, 0.407; $NaHCO_3$, 0.420; $NaH_2PO_4.H_2O$, 0.069; glucose, 1.08; and Tris buffer, pH 7.4, 1.50. Interrenal glands, 500-1000 mg per incubation, were sectioned and pre-incubated for 30-60 min in 2 ml medium/100 mg tissue, containing 1 IU ACTH/100 mg. The media from several pre-incubations were pooled for extraction. The tissue was

transferred to fresh medium with co-factors added for each 100 mg tissue: NADP, 0.65 μM; glucose-6-phosphate, 6.5 μM; glucose-6-phosphate dehydrogenase, 0.5 IU; and ACTH, 1 IU. Chemicals of the NADPH-generating system were purchased from Boehringer Mannheim Corp., New York, and ACTH from Nutritional Biochemicals Corp., Cleveland. Corticosterone in propylene glycol (3 mg/ml) was added so that the tissue:steroid ratio approximated 1000:1. Incubations were continued for 4-6 hr with co-factors replenished after 1.5 hr. A second incubation of each lot of tissue, with fresh medium and substrate, was usually carried out for a further 4-6 hr or overnight. The medium from each incubation was extracted separately.

Radioactive transformation products were prepared by incubation of 10 μc [4-^{14}C]corticosterone (New England Nuclear Corp.) with 120 mg interrenal tissue. The homogeneity of the substrate was checked by paper chromatography. The pre-incubation was carried out with all co-factors added and the pre-incubation time extended to 3 hr to deplete endogenous precursors. Incubation with substrate was continued for 4 hr.

Extraction of Steroids

Media from interrenal incubations were half-saturated with sodium sulphate before extraction with 7 volumes dichloromethane. The organic phase was washed with v/20 water and evaporated to dryness *in vacuo*. No further purification preliminary to TLC was required.

Blood plasma was extracted with dichloromethane and the extract purified for chromatography as described for salmon plasma (10).

Chromatography

Thin-layer (TLC). Thin-layer plates were prepared from silica gel previously checked for non-destruction of steroids (11). Solvent systems used in this study included the following mixtures (v/v): I- hexane:ethyl acetate (4:1); II- cyclohexane:ethyl acetate:toluene (10:10:1); III- chloroform:methanol:water (90:10:1); IV- ethyl acetate:chloroform:water (90:10:1); V- benzene:ethyl acetate (4:6); and VI- chloroform:95% ethanol (95:5).

Paper. Descending chromatography with Bush-type solvent systems (v/v) was used: B_5-benzene:methanol:water (2:1:1) and (HBM-70)-heptane:benzene:70% aqueous methanol (1:1:1).

Detection and recovery of steroid fractions. α,β-Unsaturated oxosteroids were detected on paper and thin-layer chromatograms by their absorption of ultraviolet light. Conjugated 1,4-diene-3-ones were distinguished from 4-ene-3-ones by the isonicotinic acid hydrazide spray described by Smith and Foell (12). Silica gel was removed from the plates with a vacuum elutor and extracted with dichloromethane:methanol 9:1 (v/v). Steroids were recovered from paper chromatograms by descending chromatography with methanol.

Isolation of Steroids

From interrenal incubations. Transformation products of corticosterone and, in the pre-incubates, of endogenous precursors were isolated by combined thin-layer and paper chromatography. After non-polar compounds were moved to the front of the chromatoplate by TLC in solvent system II, the plate was developed in the same direction with solvent system III. A major product more polar than cortisol was detected by its absorption of ultraviolet light. This polar fraction was further purified by paper chromatography for 18 hr in the Bush B_5 system. Methanolic eluates of the polar compound from paper chromatograms were pooled, evaporated to dryness in vacuo, and the residue dissolved in a small volume of chloroform. The chloroform extract was filtered through a fine porosity glass filter and then washed three times with v/30 water. The chloroform was removed by evaporation in vacuo and the residue dissolved in a measured volume of methanol.

From blood plasma. A purified extract of peripheral plasma from R. radiata was chromatographed bi-dimensionally on silica gel with solvent systems I and II in the first direction and with solvent system III in the second dimension as described earlier (13). A UV-light absorbing compound, blue-tetrazolium positive, occurred as a discrete spot separate from cortisol and away from the origin. This compound was eluted from TLC plates and pooled for identification studies.

Preparation of Derivatives

Acetylation. The unknown polar compound was acetylated by treating the dry compound with 20:1 acetic anhydride:pyridine for 16 hr at 37°C. The product was isolated by TLC in system IV. The rate of acetylation of reactive hydroxyl groups under these conditions was determined by the addition of 150 µl reagent to 87 µg steroid containing 9900 dpm [4-^{14}C] substituted derivative. Aliquots (20 µl) of the reaction mixture were withdrawn after 3 min, 1, 2, 3, 5 and 23 hr. The reaction was stopped by the addition of 20% ethanol. The reaction products were extracted into dichloromethane, evaporated to dryness, separated by TLC in system IV, eluted from the gel, and the radioactivity measured.

Dehydration. The steroid diacetate in glacial acetic acid was heated under reflux for 1 hr (3b). The acid was removed in vacuo and the resulting 1-dehydro-monoacetate separated by TLC in solvent system IV.

Hydrolysis of 21-acetate. The 21-ol derivative was prepared by the hydrolysis of the ketol acetate at room temperature under nitrogen according to the procedure of Sondheimer et al. (14). The reaction product was extracted into dichloromethane and purified by paper chromatography in (HBM-70) for 16 hr.

Oxidation. Selective oxidation of the 11β-hydroxyl group of the isolated polar steroid and of its derivatives was done with chromic acid as described by Bush (15).

Fluorescence in Sulphuric Acid

Fluorescence was developed in mixtures (v/v) of ethanol:sulphuric acid (70:30 and 65:35) according to the procedure of Peterson (16). The rate and sensitivity of the reaction at 25°C were compared with corticosterone as the standard steroid. Fluorescence intensities were measured with a Turner Fluorometer fitted with primary (436 mμ) and secondary (535 mμ) filters.

Spectra

Infrared spectra were recorded with a Beckman-IR10 spectrophotometer. The steroid (50-60 μg) dissolved in dichloromethane was mixed carefully with 10 mg infrared grade KBr warmed on a micro-heater. The KBr pellet was then pressed in a Carver press.

Ultraviolet spectra were recorded with a Beckman recording spectrophotometer, Model DK-2. Steroids were dissolved in methanol. The experimental procedures described by Smith and Bernstein (17) were used to prepare samples for the recording of absorption spectra in concentrated sulphuric acid.

Proton magnetic resonance spectra were recorded on a Varian A60 NMR spectrometer equipped with a C-1024 computer of average transients.

Melting Point

A No. 4015 precision melting point apparatus fitted with polaroid analyzers, Cole Parmer Instrument and Equipment Co., Chicago, Ill., was used to determine the melting point of the crystalline compounds. Values were recorded as read.

Radioactivity Measurement

Counts were made with a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3365. A concentrated liquid scintillator solution, supplied by New England Nuclear Corp., was diluted with dry toluene to a concentration of 4 g/l 2,5-diphenyloxazole and 50 mg/l p-bis [2-(5-phenyloxazolyl)]-benzene. Samples of radioactive steroid were evaporated to dryness in the counting vials. The steroid was then dissolved in 150 μl absolute ethanol and 15 ml scintillator was added. Total counts were sufficient to give a fractional error of less than 2% with a probability of 95%.

RESULTS

Extracts of interrenal incubations with corticosterone as precursor

were fractionated by TLC into three ultraviolet light absorbing bands: a polar compound with R_s = 0.6 in solvent system III (s = cortisol); usually a band of unchanged corticosterone; and a minor component, less polar than corticosterone, in the position of 11-dehydrocorticosterone. Trace amounts of two other products could be detected by an alkaline blue-tetrazolium spray. Only the polar compound was produced from endogenous precursors in quantities sufficient to be detected by UV absorption.

The transformation in vitro of corticosterone to (I) (Fig. 1) by the skate interrenal (30 incubations, 120 fish) varied quantitatively from 10% to 90% with an average value of 40%. The radioactivity recovered from the incubation of 10 μc [4-^{14}C]corticosterone (62 μg) with 120 mg interrenal tissue (R. ocellata) was measured in the total extract of the medium and in steroid fractions separated by paper chromatography. The tissue retained 12% of the radioactivity; the interrenal gland is fatty and the tissue was not extracted with solvent. From the paper chromatogram, 71% of the original radioactivity was recovered as the polar steroid, 5% as unchanged corticosterone, and 2% in the chromatographic area of 11-dehydro-corticosterone. Thus few reactions other than 1-hydroxylation of the substrate have occurred and low-yield incubates contained unchanged corticosterone rather than other products.

Preliminary spot checks on TLC of incubation media allowed the pooling of high-yield incubations for extraction. From extracts of the incubation media of a total of 4.6 mg corticosterone with 3.6 g interrenal tissue, 2.3 mg (I) were isolated by TLC and purified by paper chromatography in the Bush B_5 system for 18 hr. In this solvent system (I) gave an R_s value of 0.62 (s = cortisol). Crystallization from acetone-hexane gave a first crop of 780 μg of white needles, m.p. 201-202°; λ_{max} 242.5 mμ,

ϵ 15,600. The steroid from incubations was not purified routinely by crystallization; the residue remaining after chloroform:water partition was dissolved in methanol and the concentration determined by UV absorption.

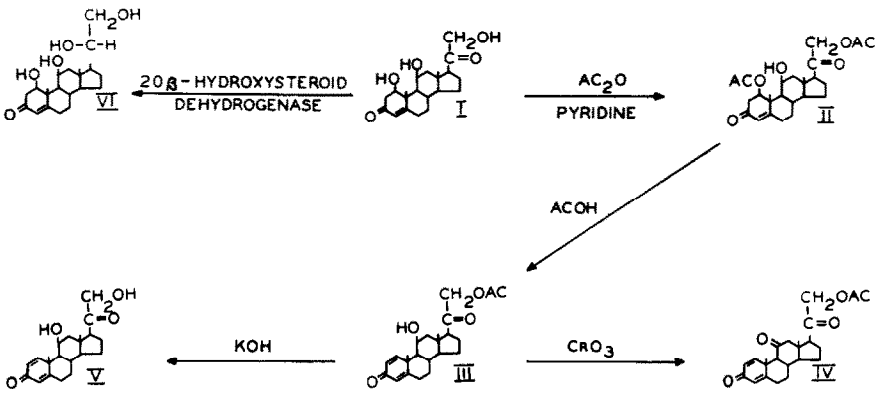

Fig. 1. Transformations of 1α-hydroxycorticosterone. I 1α-hydroxycorticosterone; II 1α-hydroxycorticosterone-1,21-diacetate; III Δ^1-corticosterone-21-acetate; IV Δ^1-11-dehydrocorticosterone-21-acetate; V Δ^1-corticosterone; VI 1α-hydroxy-20β-dihydrocorticosterone.

Preliminary studies indicated that (I) was a monohydroxylated derivative of corticosterone. A positive and immediate reaction with blue-tetrazolium indicated an α-ketol group, a negative reaction with the Porter-Silber reagent showed the absence of a hydroxyl group at C-17 or C-16α (18) and a quantitative reaction with 20β-hydroxysteroid dehydrogenase (19) confirmed the presence of a 20-carbonyl group. The Δ^4-3-ketone grouping was confirmed by the immediate reaction of the compound with isonicotinic acid hydrazide (12). A decrease in polarity and a hypsochromic effect (-5 mμ) on the absorption maximum after mild oxidation (20) indicated that the 11β-hydroxyl group of corticosterone was intact.

TABLE I

Rate of acetylation of 1α-hydroxycorticosterone at 37°C with acetic anhydride:pyridine (2:1)

Time	% Free Steroid(1)	% Monoacetate(1)	% Diacetate(1)	Total
3 min	21.8	67.8	1.8	91.4
1 hr	- (2)	60.5	17.9	78.4
2 hr	-	50.3	33.4	83.7
3 hr	-	34.2	51.8	86.0
5 hr	-	23.5	69.0	92.5
23 hr	-	0.8	78.2	79.0

(1) Fractions separated by TLC in solvent system IV.
(2) Free steroid no longer detectable by UV absorption.

A study of the rate of acetylation of (I) (see Table I) showed two reactive groups, the hydroxy group of unknown position reacting completely though much more slowly than the C-21 group.

Acetylation of (I) overnight at 37°C gave a diacetate (II) as the principal product. This derivative was isolated by TLC in system IV, purified by paper chromatography in the system (HBM-70) for 4 hr, R_s = 0.88 (s = corticosterone acetate), and then twice crystallized from acetone-hexane, m.p. 182-183°. The infrared spectrum of the diacetate showed that the steroid did not have another (tertiary) hydroxyl group.

Consideration of the ultraviolet absorption spectra of (I) (21) and of its chromatographic mobility compared with available authentic monohydroxycorticosterones (2α,2β,6β,12α,15β,16α,18 and 19) allowed the

presumptive identification of (I) as 1-hydroxycorticosterone.

Confirmation of the 1-position of the hydroxyl group was made by dehydration of the diacetate (II) and hydrolysis of the resulting C_{21}-monoacetate (III) to form the 1-dehydro derivative (V). This derivative was then identified with Δ^1-corticosterone, a compound previously described by Nobile et al. (22) and available for comparison.

The 1-dehydro compounds Δ^1-corticosterone and (V), their acetates (III), and the 11-dehydro derivatives of the steroid and ester (IV) could not be separated by TLC in systems IV, V and VI. Chromatography on paper did not separate (V) from Δ^1-corticosterone. In the (HBM-70)system overnight, the R_s values (s = corticosterone) were 0.66 for the parent compounds and 1.22 for their oxidation products. The C_{21}-monoacetate (III) and the C_{21}-monoacetate of the 11-dehydro derivative (IV) gave R_s values (s = corticosterone acetate) of 0.86 and 1.07 respectively in (HBM-70). The C_{21}-monoacetates of authentic Δ^1-corticosterone and its 11-dehydro derivative gave R_s values of 0.86 and 1.08 in the same chromatographic system.

Spectral studies of (V) and Δ^1-corticosterone confirmed the identity of the two compounds. The infrared spectra of both compounds are shown in Fig. 2. A tracing of the infrared spectrum of the parent hydroxysteroid (I) isolated from interrenal incubates is included in the figure.

The absorption spectra of (I) in concentrated sulphuric acid was measured after 10 min, 2 hr, and 24 hr. A methanolic solution of the crystalline compound equivalent to 52.2 µg was dried in a tube, 3.0 ml concd H_2SO_4 was added and the reaction allowed to proceed in the dark at 23°C. Optical density data, converted to $E^{1\%}$/cm values, are given in Table II. The absorption spectra of a methanol eluate of a paper

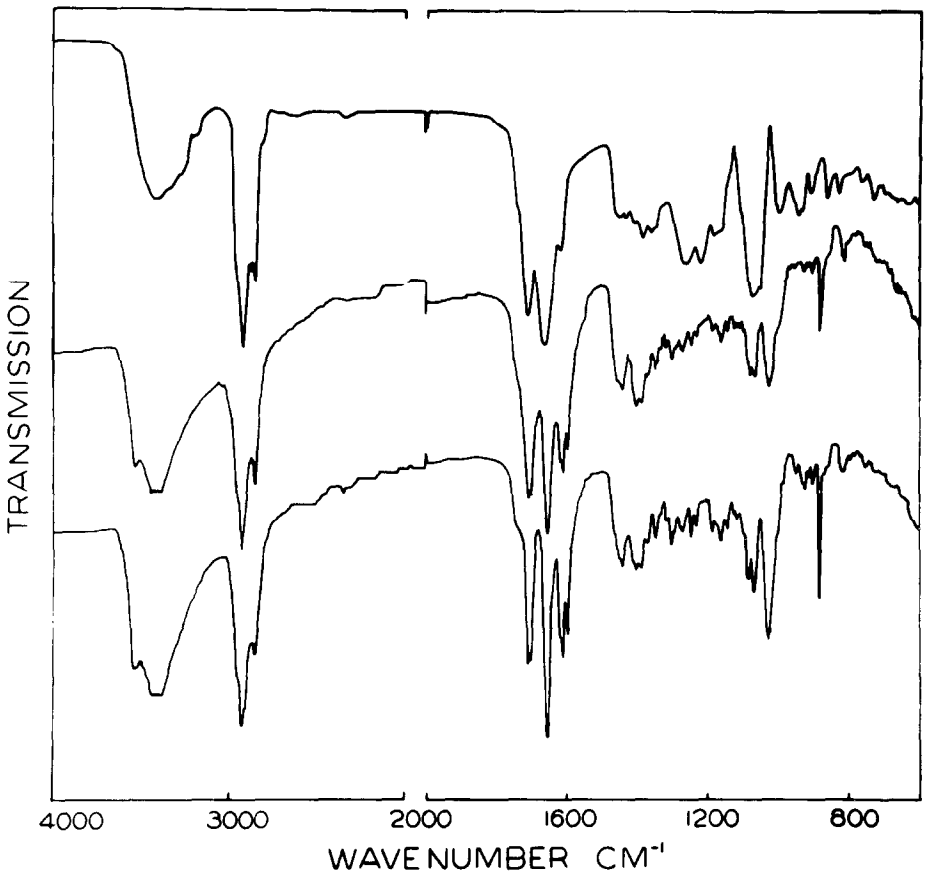

Fig. 2. Infrared spectra of 1α-hydroxycorticosterone from interrenal incubate (upper), Δ^1-corticosterone prepared from 1α-hydroxycorticosterone (middle) and Δ^1-corticosterone from MRC collection (lower).

chromatogram of (V), 40.5 μg in 3.0 ml H_2SO_4, was compared with the spectra of 42.8 μg of authentic Δ^1-corticosterone in 3.0 ml H_2SO_4. The spectra of crystalline Δ^1-corticosterone was read against a sulphuric acid blank but a papergram blank was used for (V) to minimize possible interference from paper impurities (17). Data are included in Table II.

A comparison of the pmr spectra of corticosterone and (I), and their acetate derivatives, confirmed that (I) was a hydroxy derivative of corticosterone, and showed that the hydroxyl group was in the 1α-position

TABLE II

Absorption spectra in concentrated sulphuric acid

Compound	λ_{Max} Mμ ($E^{1\%}_{1\ cm}$)[a]			λ_{Min} Mμ ($E^{1\%}_{1\ cm}$)		
	10 min	2 hr	24 hr	10 min	2 hr	24 hr
1α,11β,21-trihydroxy-pregn-4-ene-3,20-dione[b]	286 (516) 379 (96)I 478 (126) 585 (57)	241 (292)I 286 (480) 377 (209) 463 (161)	238 (408)I 283 (474) 375 (221) 463 (219)	356 (78) 420 (81) 530 (46)	250 (270) 345 (138) 430 (139)	255 (325) 345 (173) 421 (160)
11β,21-dihydroxy-1,4-pregnadiene-3,20-dione[b]		263 (285)I 281 (283)I 335 (198)I 374 (222) 461 (264)	284-286 (414) 377 (231) 462 (288)		260 (283) 272 (273) 331 (196) 405 (162)	259 (308) 339 (192) 415 (183)
1-dehydro[c] derivative of (I)		263 (301)I 278 (282)I 336 (183)I 374 (239) 461 (274)	283-285 (406) 376 (274) 462 (297)		260 (299) 273 (260) 331 (182) 407 (167)	260 (328) 336 (184) 418 (204)

[a] I denotes an inflection or plateau

[b] Crystalline compounds

[c] Derivative prepared by dehydration of 1α-hydroxycorticosterone-1,21-diacetate and hydrolysis of acetate group on C-21. Derivative purified by paper chromatography only.

of the corticosterone nucleus. The chemical shift data derived from these spectra are summarized in Table III. Coupling between the hydroxyl protons and the protons geminal to the hydroxyl groups was not observed, due to the rapid exchange of the hydroxyl protons in deuterochloroform solution.

TABLE III

Chemical shifts* of protons attached to carbon atoms at positions 1,4,11,18, and 19 in corticosterone, (I), and derivatives

Compound	19	18	4	11	1
Corticosterone	8.56	9.06	4.33	5.60	
(I)	8.55	9.05	4.25	5.45	5.63
Corticosterone monoacetate	8.50	9.07	4.34	5.56	
(I) diacetate (II)	8.49	9.05	4.25	5.88	4.58

* τ values were determined in deuterochloroform solution with tetramethylsilane as an internal reference.

The effect of hydroxyl or acetoxyl substituents on the chemical shifts of the C-18 and C-19 angular methyl protons and the C-4 olefinic proton in Δ^4-3-oxosteroids has been reported in detail by Tori and Kondo (23). These authors have also given the signal patterns, for protons geminal to the substituents, which are characteristic of the position of the substituent in the steroid nucleus.

From Table III it can be seen that the chemical shifts of the C-18 and C-19 methyl groups are virtually identical for both steroids. On the other hand the C-4 olefinic proton in (I) is shifted downfield slightly,

relative to its position for corticosterone. The spectrum of (I) contained two multiplets which were due to protons geminal to secondary hydroxyl groups. One multiplet (average τ value 5.45) was a partially resolved quartet (spacing 3 cps), identical to that observed for the 11α-proton (average τ value 5.60) in the spectrum of corticosterone. The other was a triplet (average τ value 5.63) with a spacing of 3 cps, which was partially overlapped by the signal for the C-21 methylene protons in deuterochloroform, but not in pyridine solution. Such a pattern would only be observed if the proton was geminal to a hydroxyl group in the 1α-, 6β-, or 12β-position (23). The 12β-position can be eliminated since the presence of a hydroxyl group in the 11β-position and a methyl group in the 13β-position requires that the single proton on C-12 be split into a doublet by the single proton on C-11. The 6β-position can also be eliminated since the 1,3-diaxial relationship of the 6β-hydroxyl and the 10β-methyl group would result in the latter being chemically shifted downfield relative to the corresponding methyl group in corticosterone. As mentioned above, the chemical shifts of the angular methyl groups are identical in the two steroids. Therefore, the hydroxyl group must be in the 1α-configuration, which would result in the C-4 olefinic proton experiencing a small paramagnetic shift (23) in agreement with the experimental observation.

The same conclusion may be derived from the pmr spectra of the acetate derivatives of the steroids. Once again the chemical shifts of the C-18 and C-19 methyl groups are almost the same, and the signal patterns for the protons geminal to the acetoxyl groups are identical to those described above.

The fluorescence time-curve of 1α-hydroxycorticosterone was compared with corticosterone in sulphuric acid:ethanol mixtures (Fig. 3). The

intensity of fluorescence, though lower in 65% sulphuric acid than in 70%, is sufficiently stable to permit quantitative determinations of the steroid. After 90 min, the intensity of fluorescence of 1α-hydroxycorticosterone is equal to 51.4% of that of corticosterone.

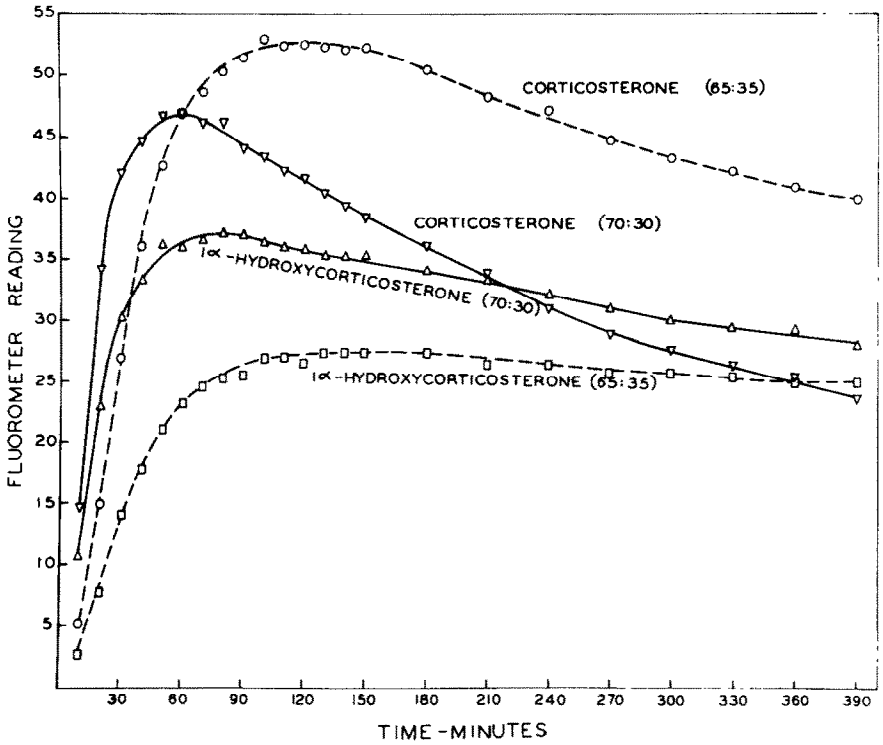

Fig. 3. Acid-fluorescence curves of corticosterone and 1α-hydroxycorticosterone.

With the identity of the corticosterone-transformation product of Raja interrenal incubates established as 1α-hydroxycorticosterone, the polar steroid present in the blood plasma was shown to be the same substance. A methanolic solution of the unknown, equivalent to 1150 ml peripheral plasma (R. radiata), was obtained from eluates of TLC plates. The quantity was estimated by UV absorption at 242.5 mμ to be 18 μg of steroid. The

blood steroid was not separable from 1α-hydroxycorticosterone on TLC in system III, R_s = 0.60 (s = cortisol). On paper the blood steroid and its derivatives behaved chromatographically as 1α-hydroxycorticosterone. In the summary below, the R values of the corresponding compound prepared from interrenal incubates are given in brackets. In the Bush B_5 system, again s = cortisol, the R_s value of the free steroid was 0.60 (0.62); the diacetate in (HBM-70) gave R_s = 0.92 (0.88); the 1-dehydro-21-acetate prepared from the diacetate gave R_s = 0.86 (0.86) with corticosterone acetate as the standard. The final paper chromatogram gave a positive $\Delta^{1,4}$-3-ketone reaction, distinct from Δ^4-3-ketone, with isonicotinic acid hydrazide (12).

To confirm the identification of the steroid in blood, the sulphuric acid chromogen was prepared by the addition of 0.2 ml sulphuric acid to the steroid isolated by TLC from 400 ml plasma. After 2 hr at room temperature the UV spectrum was read against a blank prepared from silica gel. Qualitatively, the spectrum was very similar to that of crystalline 1α-hydroxycorticosterone with maxima at 284, 365 and 465 mμ (Table II). Quantitatively, the optical densities of the peaks at 365 and 465 were too high compared to the optical density of the peak at 284. Ratios were 2.1/1.8/1.0 compared to 3.0/1.3/1.0 for the pure compound. Later studies showed that blanks taken from silica gel plates are less than ideal for UV absorption measurements.

DISCUSSION

1α-Hydroxycorticosterone was prepared biosynthetically from corticosterone in amounts sufficient to allow its identification and description.

The new steroid was identified by its chromatographic mobility, microchemical reactions, spectral studies, and finally its conversion to a known compound, Δ^1-corticosterone.

The spectrum of 1α-hydroxycorticosterone in sulphuric acid was typical of Δ^4-3-ketones and, with the dominant peak at 286 mμ, little different than the spectrum of the parent corticosterone (17). The spectrum of 1-hydroxycortisol in sulphuric acid (24) was reported to show no marked differences after 0.25 hr, 2 hr, or 24 hr. The spectrum of 1α-hydroxycorticosterone did show some differences with time (Table II) and there was a marked resemblance between the 24-hr spectrum of 1α-hydroxycorticosterone and the 24-hr spectrum of Δ^1-corticosterone. Comparison of data published for the 20-hr spectrum of Δ^1-cortisol (25) and for 1-hydroxycortisol (24) does not so readily suggest a parent-derivative relationship. The $\Delta^{1,4}$-3-ketone system is characterized by a sulphuric acid chromogen exhibiting an absorption maximum in the region 247-267 mμ with an associated band at 295-318 mμ. These characteristics sometimes disappear rapidly (25). Both these bands were observed in authentic Δ^1-corticosterone and (V). The former band occurred as a fairly sharp peak at 262 mμ at times up to 10 min, changing to a broad peak at 2 hr. The latter band had a broad maximum from 305-320 mμ at 4.5 min and this quickly assumed a gentle slope which changed to a peak at 281 mμ as observed at 2 hr.

In sulphuric acid:ethanol mixtures the fluorescence of 1α-hydroxycorticosterone, though not as intense as corticosterone, was strong compared to other steroids. In acid:ethanol (v/v) (65:35), after 3 hr, 1α-hydroxycorticosterone gave a fluorometer reading equal to 55% of the reading for corticosterone whereas readings for 11β,17α,20α,21-tetra-

hydroxypregn-4-en-3-one and for cortisol were 25% and 20% respectively as reported by Peterson (16).

Determination of the specific activity (S.A.) of [4-^{14}C] 1α-hydroxy-corticosterone, biosynthesized by interrenal tissue, involved liquid scintillation counting and it was soon obvious that, like some other polar steroids, 1α-hydroxycorticosterone was strongly adsorbed to the glass surface of the counting vials. The addition of 1% ethanol, as suggested by Kandel and Gornall (26), increased the measured S.A. from 0.087 μc/μg to 0.13 μc/μg, i.e. 33%. Samples for counting represented 0.12 μg. In a second experiment with tritiated 1α-hydroxycorticosterone, S.A. = 6.5 μc/μg, biosynthesized from [1,2-^{3}H]corticosterone, the counts were increased by 50% upon the addition of ethanol or carrier steroid.

Our interest in this compound was first aroused by two observations: 1α-hydroxycorticosterone occurred in all blood samples of Raja examined (ca. 25); and cortisol, corticosterone and aldosterone, one or more of which are normally expected to be the major corticosteroids of vertebrate classes (27), were not present in detectable amounts. Our results do not agree with the identification of steroids in the blood of some other species of Raja. The peripheral plasma of R. clavata and R. eglanteria were reported to contain 8 μg/100 ml corticosterone and 5.3 μg/100 ml cortisol respectively (28). Blood samples of the two species examined here were estimated to contain ca. 0.5-2 μg 1α-hydroxycorticosterone/100 ml plasma.

The incubation in vitro of interrenal tissue provided evidence that 1α-hydroxycorticosterone was a secretory product of the gland rather than a catabolite of corticosterone. An extract of a 40-min pre-incubation medium from 800 mg interrenal tissue of R. ocellata and added ACTH

contained 34 μg 1α-hydroxycorticosterone. Thus, the production rate from endogenous precursors was 6.4 μg/100 mg/hr. Similarly, from a 45-min pre-incubation of R. radiata tissue, with added ACTH, the production rate of 1α-hydroxycorticosterone was equal to 4.4 μg/100 mg/hr. In a study of the effect of ACTH upon corticoid synthesis in vitro of the interrenal of R. erinacea, Macchi (29) measured the production of blue-tetrazolium reducing substances and of UV-absorbing compounds. Values obtained were approximately 1 μg/100 mg/hr and 2 μg/100 mg/hr with and without the addition of ACTH, respectively. No attempt was made to identify the steroids so produced. Paper chromatographic examination of extracts of incubation medium of the interrenal glands of R. rhina (30) showed only trace quantities of corticosterone and cortisol.

Factors influencing the 1α-hydroxylase have not been studied in any detail and the reasons for the wide variation in the transformation of corticosterone are not understood. Metopirone (2-methyl-1,2-bis-(3-pyridyl)-1-propanone) was found to inhibit the enzyme (31) at a concentration of 2.2×10^{-5}M, a level comparable to that which inhibited the action of the 11-hydroxylating enzyme in mouse adrenals (32). In an attempt to increase the rate of the reaction, the incubation temperature was raised from 26°-37°C. Details of this experiment are to be published elsewhere (31) but the increase in temperature, to a norm for many mammals, resulted in complete inactivation of the enzyme. Thus this particular hydroxylase will probably not be found in warm blooded animals, and although the 1α-hydroxy group on corticosterone has the axial configuration in common with the 1β-hydroxy-5β-pregnane derivative isolated from man (8), it is unlikely that a common enzyme is operating. The extent of the occurrence of a 1α-hydroxylase among other poikilotherms is not known at the present time.

Elasmobranchs maintain the osmotic pressure of internal fluids by retention of urea and trimethlamine oxide although the sodium level is approximately one-half of that of the external marine environment. Thus, osmoregulatory problems have been solved in a manner peculiar to the group and conceivably a specialized hormonal control of the process has also been developed. The role of 1α-hydroxycorticosterone in the elasmobranch has not been established but the steroid showed mineralocorticoid activity in a rat bioassay (33).

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1. Systematic nomenclature:

Δ^1-corticosterone = 11β,21-dihydroxypregna-1,4-diene-3,20-dione
Δ^1-cortisol = 11β,17α,21-trihydroxy-1,4-diene-3,20-dione
11-dehydrocorticosterone = 21-hydroxypregn-4-ene-3,11,20-trione
Δ^1-11-dehydrocorticosterone = 21-hydroxypregna-1,4-diene-3,11,20-trione
1α-hydroxycorticosterone = 1α,11β,21-trihydroxypregn-4-ene-3,20-dione
1-hydroxycortisol = 1ξ,11β,17α,21-tetrahydroxypregn-4-ene-3,20-dione
1α-hydroxy-20β-dihydrocorticosterone = 1α,11β,20β,21-tetrahydroxypregn-4-ene-3-one

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