Journal of Steroid Biochemistry, 1973, Vol. 4, pp. 567–576. Pergamon Press. Printed in Great Britain

METABOLISM AND CONJUGATION OF CORTISOL BY VARIOUS DOG TISSUES *IN VITRO*

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(*Received* 22 *January* 1973)

SUMMARY

The metabolism and conjugation *in vitro* of [4-^{14}C]-cortisol by a variety of dog tissues have been investigated. Quantitatively, oxidation at C-11 to cortisone was the major biotransformation which occurred in all tissues studied. Reduction of C-20, primarily to 20β-dihydro metabolites and side chain cleavage to C-19 steroids was also demonstrated to an appreciable extent in all the tissues except the brain. Of the extrahepatic tissues, the kidney and adrenal cortex were the most metabolically active. Reduction of the 4–5 double bond and C-3 ketone of ring A was characteristic of the liver. 5β-Isomers were the major type of products. Small quantities of 6β-hydroxycortisol and 6β-hydroxycortisone were synthesized by the adrenal cortex and the liver. Of special interest was the isolation of 6β-hydroxy-20β-dihydro derivatives from the liver. A small fraction of ring A-reduced metabolites was conjugated with glucuronic acid in the liver, while the capacity to sulfate cortisol was noticed in the adrenal, liver, kidney, muscle and lung.

INTRODUCTION

ALTHOUGH the liver is usually accepted as being the major site for metabolism and conjugation of steroids, there is evidence that biotransformation of steroids can also occur in the extrahepatic tissues. In eviscerated rats, Berliner *et al.* [1]

The following trivial names and abbreviations are used: cortisol (F); cortisone (E); 20α-dihydrocortisol (20α-DHF): 11β, 17α, 20α, 21-tetrahydroxy-4-pregnen-3-one; 20β-dihydrocortisol (20β-DHF): 11β, 17α, 20β, 21-tetrahydroxy-4-pregnen-3-one; 20α-dihydrocortisone (20α-DHE): 17α, 20α, 21-trihydroxy-4-pregnen-3, 11-dione; 20β-dihydrocortisone (20β-DHE): 17α, 20β, 21-trihydroxy-4 pregnen-3, 11-dione; 6β-hydroxycortisol (6β-OH-F): 6β, 11β, 17α, 21-tetrahydroxy-4-pregnen-3,20-dione; 6β-hydroxycortisone (6β-OH-E): 6β, 17α, 21-trihydroxy-4-pregnen-3, 11,20-trione; 6β-hydroxy-20α-dihydrocortisol (6β-OH-20α-DHF): 6β, 11β, 17α, 20β, 21-pentahydroxy-4-pregnen-3-one; 6β-hydroxy-20α-dihydrocortisol (6β-OH-20β-DHF): 6β, 11β, 17α, 20β, 21-pentahydroxy-4-pregnen-3-one; 6β-hydroxy-20α-dihydrocortisone (6β-OH-20α-DHE): 6β, 17α, 20α, 21-tetrahydroxy-4-pregnen-3,11-dione; 6β-hydroxy-20β-dihydrocortisone (6β-OH-20β-DHE): 6β, 17α, 20β, 21-tetrahydroxy-4-pregnen-3,11-dione; tetrahydrocortisol (THF): 3α, 11β, 17α, 21-tetrahydroxy-5β-pregnene-20-one; 5α-tetrahydrocortisol (5α-THF): 3α, 11β, 17α, 21-tetrahydroxy-5α-pregnane-20-one; tetrahydrocortisone (THE): 3α, 17α, 21-trihydroxy-5β-pregnane-11,20-dione-cortol-20α: 5β-pregnane-3α, 11β, 17α, 20α, 21-pentol; cortol-20β: 5β-pregnane-3α, 11β, 17α, 20β, 21-pentol; 5α-cortol-20α or 20β: 5α-pregnane-3α, 11β, 17α, 20α or 20β, 21-pentol; cortolone-20α: 3α, 17α, 20α, 21-tetrahydroxy-5β-pregnan-11-one; cortolone-20β: 3α, 17α, 20β, 21-tetrahydroxy-5β-pregnan-11-one; 5α-cortolone: 3α, 17α, 20α, 21-tetrahydroxy-5α-pregnan-11-one; 11β-hydoxy-aetiocholanolone (11-OH-Etio): 3α, 11β-dihydroxy-5β-androstan-17-one; 11β-hydroxy-androsterone (11-OH-Andro): 3α, 11β-dihydroxy-5α-androstan-17-one; 11β-hydroxy-androstenedione (11-OH-AD): 11β-hydroxy-4-androstene-3,17-dione: 11-oxo-aetiocholanolone (11-oxo-Etio): 3α-hydroxy-5β-androstane-11,17-dione; 11-oxo-androsterone (11-oxo-Andro): 3α-hydroxy-5α-androstan-11, 17-dione; adrenosterone (Adreno): 4-androstene-3, 11, 17-trione; 6β, 11β-dihydroxy-androstenedione (6, 11-diOH-AD): 6β, 11β-dihydroxy-4-androstene-3,17-dione; 5β-hydsoxy-adrenosterone (6β-OH-Adreno): 6β-hydroxy-4-androstene-3, 11, 17-trione; aetiocholanetrione: 5β-androstan-3, 11, 17-trione; androstanetrione: 5α-androstane-3,11, 17-trione; -Ac: (steroid)-yl-monoacetate; -diAc: (steroid)-diyl-diacetate; -triAc: (steroid)-triyl-triacetate; -sulfate: (steroid)-yl-sulfate; -glucuronide: (steroid)-yl-β-D-glucopyranosiduronide.

demonstrated that cortisol was converted to 20β-dihydrocortisol, 20β-dihydrocortisone and 5-dihydrocortisol. Gold[2, 3] characterized 20β-dihydrocortisol and 20β-dihydrocortisone in the urine of hepatectomized dogs to which radioactive cortisol had been administered. A large number of studies have been carried out on cortisol metabolism *in vitro*[4]. However, the efforts to completely characterize all metabolites produced by a wide variety of tissues are few and limited to the human[5, 6] and the rat[7].

In a previous report[8], we revealed that a number of dog tissues have the capacity to sulfate cortisol *in vitro*. The present report is a sequel to that and will be devoted to characterization of unconjugated as well as glucuronic acid conjugated metabolites of cortisol formed *in vitro*.

MATERIALS AND METHODS

Materials. [4^{14}C]-cortisol (S.A. 51·8 mCi/mmol) was obtained from New England Nuclear Co., Boston, Mass. Its purity was checked by paper chromatography in the system of Bush B-5. Most of the unlabelled reference steroids were purchased from Sigma Chemical Co., St. Louis, Mo. 6β-Hydroxycortisone was obtained from Mann Research Laboratories, New York, N.Y. and 6β-hydroxycortisol was a generous gift of Dr. Seymour Bernstein, Lederle Laboratories, Pearl River, N.Y. Reduction at the C-20 ketone of 6β-hydroxycortisol and 6β-hydroxycortisone was done with sodium borohydride as described by Bradlow *et al.*[9]. The products were purified by paper chromatography in the benzene: ethyl acetate: methanol: water system (6:4:5:5, by vol) and separated into the 20α and 20β isomers in the same system, overrun twice on boric acid impregnated paper[10]. All solvents were of analytical grade and redistilled before use.

Incubation and extraction. The method has been described elsewhere[8]. Five male adult mongrel dogs, 14–17 kg each, were used. The animals were sacrified by exsanguination with the aid of a plastic catheter, which had been placed in the carotid artery 3 h prior to the experiment. The tissues were removed immediately, minced, weighed and 1500 mg portions were suspended in 20 ml of Krebs-Ringer bicarbonate buffer pH 7·4 without $CaCl_2$ containing 1·0 μCi of [4-^{14}C]-cortisol. No cofactors were added except 150 μmol of ATP. Incubation was carried out for 3 h at 36°C under 95% O_2: 5% CO_2. At the end of incubation, the tissue was homogenized in the medium and extracted twice with two volumes of acetone-ethanol (1:1, v/v) followed by one extraction with one volume of n-butanol. The extracts were combined and evaporated to dryness. The residue was redissolved in 30 ml of distilled water and the unconjugated (free) metabolites were extracted twice with 3 volumes of ethyl acetate.

Separation of free metabolites. After the evaporation of the ethyl acetate extract, the residue was subjected to several consecutive paper chromatographic steps to separate it into individual steroids (Table 1 and Fig. 1). The procedures were essentially the same as described previously[11] but a new system (S-I) was introduced for the analysis of metabolites more polar than 6β-hydroxycortisol. Chromatographic mobilities of each of the separated radioactive compounds were compared with those of the authentic reference steroids.

Separation of conjugated metabolites. The conjugated metabolites remaining in the aqueous phase were extracted on an Amberlite XAD-2 column[12] and separated into glucuronide and sulfate conjugates by means of high voltage elec-

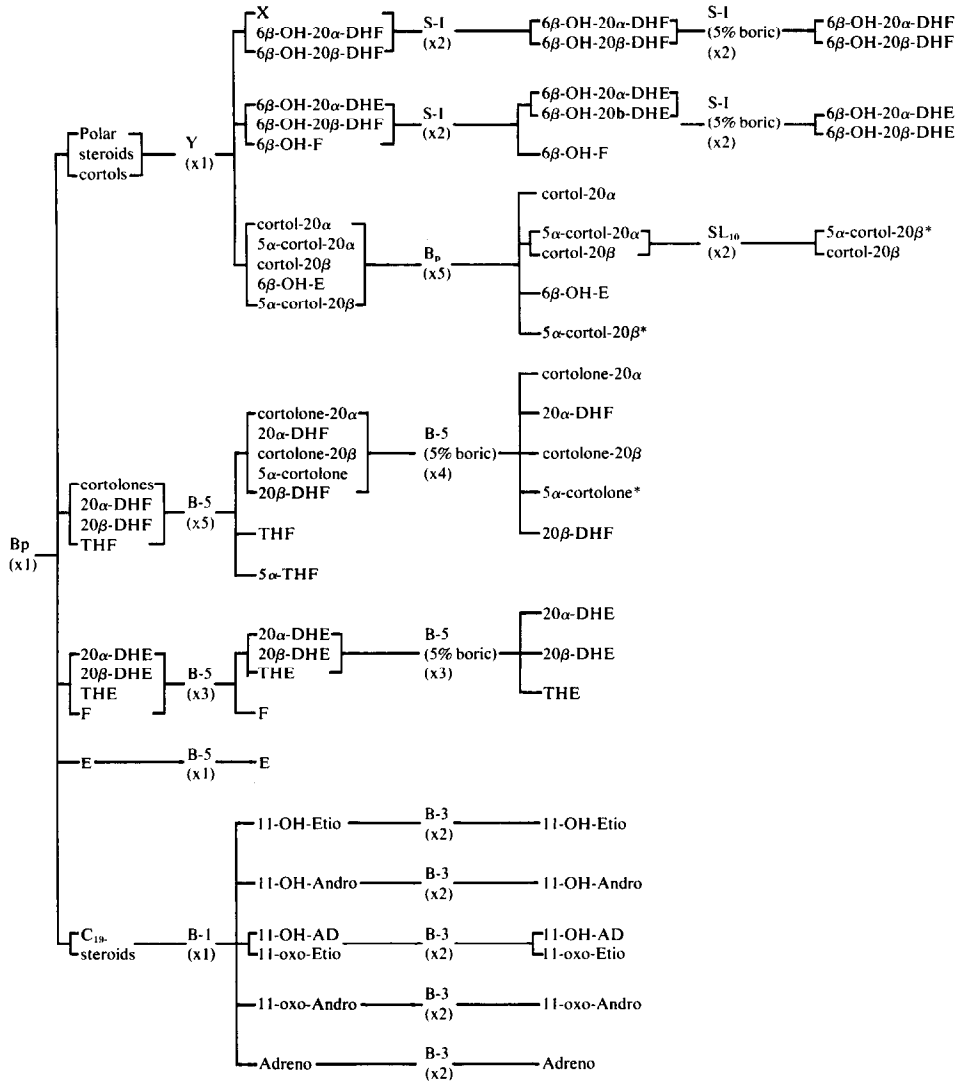

Fig. 1. Paper chromatographic separation of free cortisol metabolites. For designation of chromatographic systems, see Table 1; numbers in parentheses indicate the length of the run, e.g. (x3) = 3 times single length run. For steroid abbreviation and nomenclature see footnote in the text. *Indicates no authentic standards available.

trophoresis as described by Kornel[13]. The liver was the only tissue which yielded a glucuronide peak after the first pH 2·4 electrophoresis. All glucuronide peaks were pooled and purified by an additional electrophoresis at pH 6·4. This was eluted, evaporated and the residue was dissolved in water buffered with acetate buffer (pH 4·5) and hydrolyzed with β-glucuronidase (Tokyo-zoki Co., Tokyo, 2 ml, 13000 U/ml) for 36 h. The liberated steroids were extracted with ethyl acetate and separated by the same paper chromatographic systems as used for the free metabolites. The analysis of sulfate conjugate has been reported previously [8].

Identification of individual steroids. For free metabolities, the chromatograph-

Table 1. Paper chromatographic systems

System designation	Solvents (proportions by volume)
Y	ethyl acetate/chloroform/methanol/water (25:75:50:50)
S-I	benzene/ethyl acetate/methanol/water (50:40:50:50)
(5% boric)	(same as S-I) before application, paper was dipped in 5% boric acid and dried in hood.
Bp	benzene/chloroform/methanol/water (50:50:50:50)
B-5	benzene/methanol/water (1,000:525:475)
(5% boric)	(same as B-5) before application, paper was dipped in 5% boric acid and dried in hood.
SL_{10}	toluene/tert-butaol/methanol/0·02 M boric buffer (pH 9·0) (170:40:30:100) (paper preimpregnated in the boric buffer and dried)
B-1	petroleum ether/toluene/methanol/water (25:25:35:15)
B-3	petroleum ether/benzene/methanol/water (33:17:40:10)

ically identical compounds obtained from all incubates were pooled together and rechromatographed in the appropriate system. The eluted steroid was identified by the method of Berliner and Salhanick[14], which may be more time-consuming than recrystallization, but very useful when dealing with small amounts of radioactivity or with limited availability of carrier steroids[15]. Details of the procedures were described in a previous paper[11]. An appropriate non-radioactive carrier was mixed with the radioactive steroid, and the mixture was rechromatographed on paper. The specific activity (d.p.m./μmol) was determined by radioactivity counting and chemical quantitation of an aliquot of the eluate. The remaining part was subjected to derivative formation either by acetylation or by oxidation, or both. Sodium bismuthate was used for oxidation of the dihydroxyacetone side chain, periodate for the glycerol side chain of C_{21} metabolites and chromic acid for 11β-hydroxyl group of C_{19} steroids. After chromatography, the specific activity of the derivative was determined and compared with that of the parent compound. They should agree within $\pm$ 5% of the mean value (Table 2).

Radioactivity measurement and quantitative estimation. A Beckman liquid scintillation counting system (LB-222B) was used. The samples were counted either in toluene containing 4·0 g PPO and 50 mg POPOP per 1000 ml or in the same solution supplemented with 10% by volume of Biosolve (BBS-II, Beckman Instrument INC. Fullerton, Ca.). The total number of counts was determined at each successive step in the procedure and corrected for counting efficiency by automatic external standardization. At each step, the recovery of steroid was corrected to 100 per cent in terms of loss of total counts and the radioactivity in individually isolated metabolites was expressed as the percentage of the initial free or glucuronide conjugated radioactivity.

RESULTS

Table 3 shows the distribution of radioactivity in the free and conjugated fractions of tissue extracts. Most of the radioactivity remained in the free fraction. Glucuronide conjugates were formed to a measurable extent only in the

Table 2. Identification of metabolites of [4-^{14}C]-cortisol isolated from tissue

Postulated steroid metabolite[1]	Nonradioactive carrier added	Chromatography[2]	Specific activity d.p.m./μmol	Chemical reaction applied	Derivative formed	Chromatography of derivative[3]	Specific activity d.p.m./μmol
6β-OH-20β-DHF	6β-OH-20β-DHF	S-I(5% boric)(x2)	155	acetylation	6β-OH-20β-DHF-triAc	B-1 (x1)	136
				KIO_4 oxidation	6, 11-diOH-AD	B-5 (x1)	149
	6β-OH-F		—	$NaBiO_3$ oxidation	6, 11-diOH-AD	B-5 (x1)	—
6β-OH-20β-DHF	6β-OH-20β-DHE	S-I(5% boric)(x2)	837	acetylation	6β-OH-20β-DHE-triAc	B-1 (x1)	801
				KIO_4 oxidation	6β-OH-Adreno	B-5 (x1)	849
	6β-OH-E		—	$NaBiO_3$ oxidation	6β-OH-Adreno	B-5 (x1)	—
6β-OH-F	6β-OH-F	S-I (x2)	311	acetylation	6β-OH-F-diAc	B-1 (x1)	291
				$NaBiO_3$ oxidation	6, 11-diOH-AD	B-5 (x1)	302
6β-OH-E	6β-OH-E	S-I(x1)	366	acetylation	6β-OH-E-diAc	B-1 (x1)	341
				$NaBiO_3$ oxidation	6β-OH-Adreno	B-5 (x1)	335
cortol-20α	cortol-20α	SL_{10}(x3)	665	KIO_4 oxidation	11-OH-Etio	B-3(x2)	673
5α-cortol-20α	—	SL_{10}(x2)	—	KIO_4 oxidation	11-OH-Andro	B-3 (x2)	—
cortol-20β	cortol-20β	SL_{10}(x2)	982	KIO_4 oxidation	11-OH-Etio	B-3 (x2)	953
5α-cortol-20β	—	SL_{10}(x2)	—	KIO_4 oxidation	11-OH-Andro	B-3 (x2)	—
cortolone-20α	cortolone-20α	B-5(5% boric)(x4)		acetylation	cortolone-20α-triAc	B-3 (x1)	321
					20α-DHF-diAc	B-3 (x1)	459
20α-DHF	20α-DHF			CrO_3 oxidation	etiocholanetrione	B-3 (x1)	301
					Adreno	B-3 (x1)	480
cortolone-20β	cortolone-20β	B-5(5% boric)(x4)	798	KIO_4 oxidation	11-oxo-Etio	B-3 (x1)	741
5α-cortolone[4]	—	B-5(5% boric)(x4)	—	KIO_4 oxidation	11-oxo-Andro	B-3 (x1)	—
20β-DHF	20β-DHF	B-5(5% boric)(x4)	1211	KIO_4 oxidation	11-OH-AD	B-3 (x1)	1132
THF	THF	B-5(x5)	556	acetylation	THF-diAc	B-3 (x1)	572
5α-THF	5α-THF	B-5(x5)	301	acetylation	5α-THF-diAc	B-3 (x1)	331
20α-DHE	20α-DHE	B-5(5% boric) (x3)	226	KIO_4 oxidation	Adreno	B-3 (x1)	200
20β-DHE	20β-DHE	B-5(5% boric)(x3)	448	KIO_4 oxidation	Adreno	B-3 (x1)	417
THE	THE	B-5(x3)	506	acetylation	THE-diAc	B-3 (x1)	478
F	F	B-5(x3)	3122	acetylation	F-21-Ac	B-3 (x1)	3028
E	E	B-5(x1)	1365	acetylation	E-21-Ac	B-3 (x1)	1279
11-OH-Etio	11-OH-Etio	B-1(x1)	221	CrO_3 oxidation	etiocholanetrione	B-3 (x1)	241
11-OH-Andro	11-OH-Andro	B-1(x1)	198	CrO_3 oxidation	androstanetrione	B-3 (x1)	211
11-OH-AD	11-OH-AD	B-1(x1)	877	CrO_3 oxidation	Adreno	B-3 (x1)	844
11-oxo-Etio	11-oxo-Etio	B-1(x1)	312	CrO_3 oxidation	etiocholanetrione	B-3 (x1)	336
11-oxo-Andro	11-oxo-Andro	B-1(x1)	266	CrO_3 oxidation	androstanetrione	B-3 (x1)	244
Adreno	Adreno	B-1(x1)	396	CrO_3 oxidation	Adreno	B-3 (x1)	412

[1]For steroid abbreviations and nomenclature see footnote in the text.

[2]For designation of chromatographic systems, see Table 1: numbers in parentheses indicate the length of the run, e.g., (X3) = 3 times single length run.

[3]Chromatographic mobility of radioactive derivative identical with that of steroid standard listed in the preceding column.

[4]Separation of 20α- and 20β-epimers was not carried out.

Table 3. Radioactivity of free and conjugated steroid fractions in various dog tissue incubates[1]

Tissue	No. of Animals	Free[2]	Conjugates[3] Glucuronide	Sulfate[4]
Adrenal	5	98·3 ± 4·1	—	1·7 ± 0·2
Kidney	5	94·2 ± 5·9	—	5·8 ± 1·2
Diaphragm	5	99·3 ± 3·8	—	0·7 ± 0·1
Lung	5	99·4 ± 4·7	—	0·6 ± 0·1
Spleen	3	100·0	—	—
Intestine	3	100·0	—	—
Brain	3	100·0	—	—
Liver	5	96·8 ± 3·4	1·5 ± 0·4	1·7 ± 0·7

[1]Results are expressed as per cent of radioactivity initially added and corrected for methodological losses. Mean ± S.D.

[2]Radioactivity recovered by ethyl acetate extraction.

[3]Radioactivity after separation of conjugates by means of high voltage electrophoresis.

[4]Results were reported previously (see Ref. [5]).

liver, while, as reported previously[8], the sulfation of cortisol occurred in a number of tissues such as the adrenal, kidney, diaphragm, lung as well as in the liver.

Analysis of the free metabolites is shown in Table 4. In most of the extrahepatic tissues, oxidation of cortisol to cortisone was the most predominant reaction, followed by the oxidative removal of the side chain to C_{19} steroids. The kidney was most active in carrying out the reactions. Reduction of the C-20 ketone group also occurred in all tissues except the brain. The adrenal had the highest capacity and both 20α- and 20β- dihydrocortisol were isolated. In the other tissues, however, only the 20β-isomer was detected. 20-Dihydrocortisone was a minor product and was found to be exclusively the 20β-form. Conversion to 6β-hydrocortisol and 6β-hydroxycortisone occurred only in the adrenal. The liver carried out all these reactions. Moreover, it had a characteristic capacity to reduce the 4-ene structure and the C-3 ketone of ring A. Both 5α- and 5β- isomers were found in cortol, cortolone, tetrahydrocortisol and C_{19} metabolites and the ratio of 5β/5α compounds was 2·3. The 3-epimers could not be resolved by the chromatographic systems used. As in the other tissues, reduction of the C-20 ketone yielded mainly 20β-dihydro-compounds. 6β-Hydroxylation also took place but 6β-hydroxycortisol and 6β-hydroxycortisone were seen only in minute amounts. An interesting finding was the presence of relatively large quantities of metabolites more polar than 6β-hydroxycortisol. Chromatography (Y,S-I and S-I on boric acid treated paper) gave three radioactive peaks and comparison of their mobilities with those of the authentic compounds suggested that the major product was 6β, 17α, 20β, 21-tetrahydroxy-4-pregnen-3, 11-dione and the minor one was 6β, 11β, 17α, 20β, 21-pentahydroxy-4-pregnen-3-one. Their identity was confirmed by reverse isotope dilution followed by derivative formation. The specific activities of the parent compounds and their acetylation and periodate-oxidation products were in close agreement. An additional evidence of identity was given by the fact that bismutate oxidation of the compounds together with either 6β-hydroxycortisone or 6β-hydroxycortisol yielded one radiochemical

Table 4. Free metabolites of [4-^{14}C]-cortisol produced by various dog tissues[1]

	Adrenal	Kidney	Diaphragm	Lung	Spleen	Intestine	Brain	Liver
No. of animals								
Steroids[2]	5	5	5	5	3	3	3	5
X[3]	—	—	—	—	—	—	—	1·2 ± 0·4
6β-OH-20β-DHF	0·2	—	—	—	—	—	—	0·8 ± 0·3
6β-OH-20β-DHE	—	—	—	—	—	—	—	3·8 ± 0·8
6β-OH-F	1·8 ± 0·7	—	—	—	—	—	—	0·2 ± 0·1
6β-OH-E	0·3 ± 0·1	—	—	—	—	—	—	0·2 ± 0·1
cortol-20α	—	—	—	—	—	—	—	1·6 ± 0·7
cortol-20β	—	—	—	—	—	—	—	2·8 ± 0·9
5α-cortol-20β	—	—	—	—	—	—	—	1·9 ± 0·9
cortolone-20α	—	—	—	—	—	—	—	1·9 ± 1·0
cortolone-20β	—	—	—	—	—	—	—	2·8 ± 1·1
5α-cortolone[4]	—	—	—	—	—	—	—	1·8 ± 1·0
20α-DHF	1·9 ± 0·8	—	—	—	—	—	—	2·2 ± 0·9
20β-DHF	11·5 ± 2·5	3·7 ± 1·3	2·4 ± 0·5	4·9 ± 1·0	2·7 ± 1·4	2·1 ± 0·4	—	4·8 ± 1·2
THF	—	—	—	—	—	—	—	3·5 ± 0·9
5α-THF	—	—	—	—	—	—	—	2·3 ± 1·2
THE	—	—	—	—	—	—	—	3·2 ± 0·7
20α-DHE	—	—	—	—	—	—	—	0·6 ± 0·2
20β-DHE	1·9 ± 0·9	1·8 ± 0·2	0·5 ± 0·1	1·0 ± 0·3	0·4 ± 0·2	0·5 ± 0·2	—	1·8 ± 0·4
F	66·9 ± 7·6	59·1 ± 8·2	78·8 ± 6·5	72·5 ± 7·7	76·7 ± 5·2	87·5 ± 6·9	82·8 ± 8·1	25·3 ± 5·1
E	9·3 ± 1·1	23·5 ± 3·8	12·3 ± 5·4	16·3 ± 1·6	14·3 ± 4·2	6·1 ± 2·8	17·2 ± 5·1	24·2 ± 3·9
11-OH-Etio	—	—	—	—	—	—	—	1·9 ± 0·6
11-OH-Andro	—	—	—	—	—	—	—	1·1 ± 0·2
11-OH-AD	5·2 ± 1·8	9·6 ± 3·0	4·7 ± 1·9	4·5 ± 2·6	4·7 ± 1·8	2·9 ± 1·1	—	5·8 ± 1·3
11-oxo-Etio	—	—	—	—	—	—	—	1·2 ± 0·4
11-oxo-Andro	—	—	—	—	—	—	—	1·0 ± 0·4
Adreno	1·0 ± 0·3	2·3 ± 0·9	1·3 ± 0·7	0·8 ± 0·3	1·2 ± 0·4	1·0 ± 0·4	—	2·1 ± 0·6

[1] Results are expressed as per cent of total free radioactivity extracted by ethyl acetate and corrected for procedural losses. Mean ± S.D.

[2] For steroid abbreviations and nomenclature see footnote in the text.

[3] X unidentified metabolites(s).

[4] Separation of 20α- and 20β- epimers was not carried out.

spot of product on the subsequent paper chromatography. No 6β-hydroxy-20α-dihydro isomers were found in significant amounts. The third, very polar compound(s) (X) remained unidentified.

Analysis of the glucuronide conjugated metabolites produced by the liver is given in Table 5. All isolated steroids had the ring A-reduced structure. Because of the small amount of glucuronide conjugate available, separation of cortol and cortolone into the four isomers, and isolation of individual C_{19} metabolites were not carried out.

DISCUSSION

The results indicate that the dog adrenal cortex is capable of transforming its own secretory product, cortisol. The reduction of the C-20 ketone and oxidation of the C-11 hydroxyl group were substantial. Though both 20α- and 20β-dihydro isomers were formed, the latter was predominant. The 20-reduced compounds have been isolated from several sources, including the beef, hog [18] and human

Table 5. Glucuronide metabolites of[4-^{14}C]-cortisol produced by dog liver *in vitro*

Steroid liberated by β-glucuronidase hydrolysis[1]	per cent radioactivity[2]	Chromatographic systems used for comparing with authentic standard[3]	Chemical reaction	Derivative formed	Chromatographic systems used for comparing with authentic standard derivative[3]
cortols[4]	27·8 ± 6·6	B_p (x6)	$NaBiO_3$ oxidation	11-OH-Etio	B-1 (x1)
cortolones[5]	23·2 ± 4·5	B_p (x3)	$NaBiO_3$ oxidation	11-oxo-Etio	B-1 (x1)
THF	26·1 ± 7·8	B-5 (x4)	$NaBiO_3$ oxidation	11-OH-Etio	B-1 (x1)
5α-THF	10·4 ± 4·7	B-5 (x4)	$NaBiO_3$ oxidation	11-OH-Andro	B-1 (x1)
THE	8·5 ± 2·1	B-5 (x3)	$NaBiO_3$ oxidation	11-oxo-Etio	B-1 (x1)
C_{19} steroids[6]	4·0 ± 2·1	—	—	—	—

[1]For steroid abbreviations and nomenclature see footnote in the test.

[2]Per cent of total glucuronide conjugate radioactivity eluted from high voltage electrophoretogram and corrected for procedural losses. Mean ± S.D. (5 animals)

[3]For designation of chromatographic systems see Table 1; numbers in parentheses indicate the length of the run, e.g. (x3) = 3 times single length run.

[4]Separation of epimers was not carried out.

[5]Separation of epimers was not carried out.

[6]No further characterization was carried out.

[19, 20] adrenals and the adrenal venous plasma of the human[19] as well as of the dog[17]. The *in vitro* conversion of cortisol of the 20-reduced metabolites has been demonstrated in the human adrenal[6]. The adrenal synthesis of 11β-hydroxyandrostenedione and the significance of cortisol as its precursor are now well established[21]. 6β-Hydroxylation of cortisol was the minor but definite metabolic pathway. The adrenal secretion of 6β-hydroxycortisol in the dog was suggested[17] and the synthesis from cortisol has been demonstrated in the human and guinea pig adrenals[6, 16]. In contrast to the adrenals of both species [6, 16], no 2α-hydroxylation product was detected in the dog.

The kidney has been regarded as the most active extrahepatic tissue in cortisol metabolism[5, 7, 22, 23], but the preferred route of transformation may differ with the species. The present study showed that the dog kidney had high capacity to oxidize the 11β-hydroxyl group and to cleave the side chain to the C-19 compounds. The C-20 reduction also occurred to an appreciable extent, but exclusively to the 20β-isomers. The same reactions took place in the muscle, spleen, lung and intestine, but to a lesser extent. Cortisone was the only product detected in the dog brain. This confirms the earlier observations with the same species[24, 25], whereas the other minor reactions could occur in the rat brain [7, 25, 26].

The ability to reduce ring A was limited to the liver. The ring A reduced products constituted 27·2% of total free metabolites and the 5β-configuration was predominant. The dog, in this respect, is similar to man[27], while the rat liver contains mainly 5α-steroid hydrogenase and a definite sex difference in the enzyme activity has been known to exist[28, 29]. The oxidation of the 11β-hydroxyl group, reduction of the C-20 ketone and oxidative cleavage of the side chain were observed in ring A intact and ring A reduced forms. Hepatic conversion to 6β-hydroxycortisol and 6β-hydroxycortisone was demonstrated but the amounts were less than in the adrenals. This is in contrast to the finding in man[6].

Quite unexpected was the finding of a relatively high yield of 6β-hydroxy-20β-dihydro metabolites. To our knowledge, this type of compound has been detected only in the human pregnancy urine [30], liquor amnii [31] and the urine of patients with Cushing's syndrome [32, 33], but *in vitro* formation has never been reported. The pathway from cortisol to the metabolites has not been established at present, but the studies *in vivo* in Cushing's syndrome suggested that at least a part of the compounds originate from plasma-borne 20β-dihydrocortisol [32, 33]. Direct conversion from 20β-dihydrocortisol and 6β-hydrocortisol has to be compared.

Only 1·5% of total radioactivity incubated with the liver was recovered as glucuronide conjugates, and no other tissues formed this conjugate. This is in distinct contrast to sulfoconjugation which occurred in a variety of tissues to a greater extent [8]. The poor hepatic glucuronylation is probably due to the fact that the conjugation takes place primarily at the C-3 hydroxyl group of ring A reduced metabolites [34], while sulfation occurs at the C-21 position of intact cortisol [5], and *in vitro* the reaction is largely dependent on the amount of uridine diphosphoglucuronic acid [35] which was not added in the present system. Gold [2, 3] demonstrated that the ring A reduced metabolites such as cortols (3α,5β and 3β,5α), cortolone (3α,5β) and tetrahydrocortisols (3α,5β and 3β,5α) were excreted in the form of glucuronide conjugates in the intact dog, while no glucuronides were found in the urine of hepatectomized dog, indicating that hepatic conjugation with glucuronic acid is an important process of cortisol metabolism occuring *in vivo* in the dog.

It is obvious that all incubation studies have common disadvantages. They could test the ability of a tissue to convert one steroid to another, but they do not allow for removal of conversion products which could cause the metabolic pattern to be altered [4]. Furthermore, the degree of tissue integration and the availability of cofactors are known to have a great influence upon the process [7]. Thus, the conditions prevailing are far from physiological. Nevertheless, the results obtained by us showed that certain biotransformations and sulfoconjugation of cortisol can be carried out by various tissues, which might be important for the tissue to regulate the "effective hormone level" *in situ*. On the other hand, the ring A reduction and subsequent conjugation with glucuronic acid by the liver, by its great capacity, serve primarily for the regulation of "cortisol pool" in the whole body.

ACKNOWLEDGEMENTS

We are grateful to Miss Fumiko Kaneto for her skilled assistance and to Dr. Lydia Torio and Mrs. Nobuko Okino for their help in manuscript preparation. This work was partially supported by a grant-in-aid for scientific research from the Japanese Ministry of Education (757093).

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