BBA 4098

THE BIOSYNTHESIS *IN VITRO* OF RADIOACTIVE CORTICOSTEROIDS FROM [4-^{14}C]PROGESTERONE BY ADRENAL SLICES OF THE DOMESTIC DUCK (*ANAS PLATYRHYNCHOS*)

THOMAS SANDOR* AND ANDRÉ LANTHIER

Laboratoire d'Endocrinologie, Hôpital Notre-Dame and Département de Médecine, Université de Montréal, Montréal (Canada)

(Received December 10th, 1962)

SUMMARY

Adrenal tissue slices originating from two female domestic ducks were incubated in a Krebs–Ringer medium using [4-^{14}C]progresterone as precursor. After extracting the medium with organic solvents, the crude extract was subjected to extensive fractionation in paper partition systems. The transformation products isolated were identified by the carrier technique. The following is a list of the major [^{14}C]corticosteroids isolated and identified: aldosterone, 18-hydroxycorticosterone-20 → 18 cyclic hemiketal, corticosterone, cortisol and 11-deoxycorticosterone. 18-Hydroxy-11-deoxycorticosterone, though looked for, was not found in the mixture. The transformation rates (expressed as per cent of the precursor added) were the most important for 18-hydroxycorticosterone (15.33%); corticosterone (11.43%) and aldosterone (6.85 %).

INTRODUCTION

In recent years, a growing interest has been shown in the comparative aspects of adrenal steroidogenesis in vertebrates other than mammals. In most studies, analysis of the adrenal effluent or peripheral blood constituted the experimental approach, but in a few instances, adrenal steroidogenesis was investigated by techniques *in vitro*.

These studies included the classes of birds[1,2], reptiles[3], amphibia[4–7] and teleost fishes[8,9]. It is fully appreciated that the results of incubation *in vitro* of adrenal tissue do not necessarily reflect the adrenal cortical secretion pattern *in vivo*. However, this method, by its virtue of relative simplicity and rapidity, constitutes a very good preliminary approach to the functional study of these glands.

In the present paper, the transformation *in vitro* of [4-^{14}C]progesterone by surviving domestic duck (*Anas platyrhynchos*) adrenal slices was studied. The duck

Abbreviations: B, corticosterone; 18OH B, 18-hydroxycorticosterone-20 → 18 cyclic hemiketal.

* Medical Research Associate of the Medical Research Council of Canada.

was chosen as tissue donor following the recent work of HOLMES *et al.*[10] and PHILIPS *et al.*[11,12] on the adrenal dependence of the extrarenal electrolyte excretory mechanism in this bird. It was considered important to establish the nature of adrenal cortical secretion in this animal, especially as only limited information is available on the adrenal secretory pattern in birds.

EXPERIMENTAL

The adrenals of two young female ducks of the Pekin white variety were used. The animals were decapitated, the adrenals removed, dissected and cut into 0.5-mm slices with a Steadie–Riggs hand microtome. The total tissue utilized had a weight of 175 mg. The tissue was incubated in a Krebs–Ringer–phosphate[13] medium at 40° for 4 h. As substrate, chromatographically pure [4-^{14}C]progesterone was utilized in the amount of $1.64 \cdot 10^6$ counts/min (specific activity: 26.2 μC/μmole). The incubation was terminated by freezing. The medium was separated from the tissue and extracted first with ethyl acetate (4 vol.) followed by extraction with chloroform (4 vol.). The combined extracts were taken down to dryness under reduced pressure and the dry residue fractionated by paper chromatography.

Chromatography

The first system utilized was that of *tert.*-butanol – isooctane – water (1:2:1.8). The paper was scanned for radioactivity and the radioactive zones eluted. The eluted zones were further analyzed by serial paper chromatography. The composition of the systems used together with the conditions of the chromatography have been described previously[14,15].

Chromatographic paper strips were scanned for radioactivity in an Actigraph II C100B 2pi radiochromatogram scanner (Nuclear Chicago Corp., Des Plaines, Ill. (U.S.A.)). Carrier steroids were visualized on paper with the aid of an ultraviolet-light source. (Maximal emission: 254 mμ.)

Quantitative measurement of carrier steroids and of the radioactivity

Carrier steroids were quantitated by ultraviolet spectrophotometry (Beckman Model-DU quartz spectrophotometer, Beckman Instruments, Inc., Fullerton, Calif. (U.S.A.)) using the formula:

$$\text{Concentration } (\mu\text{g/ml}) = \frac{\text{absorbancy x molecular weight x 1000}}{\text{molar extinction coefficient}}$$

and by the alkaline blue-tetrazolium reaction[16]. Counting of radioactivity was performed on a liquid scintillation spectrometer (Tricarb, Model 314EX, Packard Instrument Company, La Grange, Ill. (U.S.A.)) with an average efficiency of 65%. For specific activity calculations, sufficient counts were accumulated to give a standard error of less than $\pm 2\%$.

Criteria of radiochemical purity

For identification of radioactive transformation products, the carrier technique of BERLINER AND SALHANICK was used[17]. Identity of a carrier substance with the radioactive material was admitted, when three consecutive specific activities had

a coefficient of variation of less than $\pm 5\%$. Whenever possible, the different identification steps included the formation of derivatives. The following reactions were used for formation of derivatives: acetylation with acetic anhydride in pyridine at room temperature[18], oxidation with HIO_4[18], oxidation with chromium trioxide–pyridine complex[19] and oxidation with sodium bismuthate[20].

RESULTS

The following steroids were isolated and identified from the incubation mixture: 18-hydroxycorticosterone-20 —> 18 cyclic hemiketal, aldosterone, corticosterone, cortisol and 11-deoxycorticosterone.

Identification of individual steroids

Aldosterone: On the initial paper system of *tert.*-butanol–isooctane–water, aldosterone migrated in mixture with 18-hydroxycorticosterone. However, it could be separated from the latter on the system benzene–acetone–water. After this second chromatography, 185 μg of synthetic *d*-aldosterone was added to the ^{14}C material. On further manipulation the carrier did not seperate from the biosynthetic material. Procedures and specific activities are shown in Table I.

TABLE I

IDENTIFICATION OF BIOSYNTHETIC [4-^{14}C]ALDOSTERONE

For the exact composition and origin of paper chromatographic systems see refs. 14 and 15.

Step	*Chromatographic system*	*Steroid*	*μg*	*Total counts/min*	*Specific activity (counts/min per μmole)*
1	*Tert.*-butanol–isooctane–water	—	—	347 000	—
2	Benzene–acetone–water	Aldosterone	175	108 000	—
3	Benzene–50% aq. methanol	Aldosterone	154	112 725	263 976
4	Benzene–acetone–water	Aldosterone	142	100 370	251 152
5	Toluene–isooctane–70% aq. methanol	Aldosterone-18,21-diacetate	154	85 160	246 636
6	Toluene–75% aq. methanol	Aldosterone-18,21-diacetate	129	77 740	267 811
7	Light petroleum–toluene–70% aq. methanol	Aldosterone-18,21-diacetate	118	65 309	245 960

Mean specific activity (Steps 3–7) = 255 107 ± 8625 counts/min/μmole (± 3.38%)

18-Hydroxycorticosterone-20—> 18 cyclic hemiketal (18OH B): This substance as pointed out earlier[15] is very unstable. In view of this, after mixing the biosynthetic ^{14}C substance with 20 μg of synthetic *dl*-18-hydroxycorticosterone-20 —> 18 cyclic hemiketal, it was transformed to the stable 11β,18-dihydroxy 3-keto-4-etienic acid lactone. This substance and its 11-oxo derivative are not prone to tautomerization on repeated chromatography. In view of the limited amount of carrier available, an additional step of identification was introduced. Synthetic 18-hydroxy-3,11-diketo-4-etienic acid lactone was prepared from *d*-aldosterone by the reaction sequence described by HAM *et al.*[21] and ULICK AND KUSCH[6] and this material was used for further

TABLE II

COMPARISON OF THE BIOSYNTHETIC ^{14}C MATERIAL WITH SYNTHETIC *dl*-18-HYDROXYCORTICOSTERONE FOLLOWING CHROMATOGRAPHIC PURIFICATION AND PREPARATION OF DERIVATIVES

Step	*Substance*	*μg*	*Chromatographic system*	*Total counts/min*	*Specific activity (counts/min per μmole)*
1	—	—	*Tert.*-butanol–isooctane–water	347 000	—
2	—	—	Benzene–acetone–water	255 180	—
3	18OH B	20	(Before chromatography)	255 180	4623 861
4	18OH B	18.3	*Tert.*-butanol–isooctane–water	252 230	4994 980
5	Cpd. I*	16.2	Toluene–isooctane–70% methanol	228 178	4653 704
6	Cpd. II**	10.3	Toluene–isooctane–70% methanol	166 540	5299 586
7	Cpd. II**	138.22	(Before chromatography)	166 540	397 642
8	Cpd. II**	123.22	Toluene–isooctane–70% methanol	153 645	409 987

Mean specific activity (Steps 4–6) = 4982 753 ± 226 036 counts/min/μmole (± 4.5%)

Mean specific activity (Steps 7–8) = 403 314 ± 5646 counts/min/μmole (± 1.4%)

* HIO_4 oxidation product of 18OH B = 11β,18-dihydroxy-3-keto-4-etienic acid lactone.
** CrO_3–pyridine oxidation product of Cpd. I = 18-hydroxy-3,11-diketo-4-etienic acid lactone.

TABLE III

IDENTIFICATION OF BIOSYNTHETIC [4-^{14}C]CORTICOSTERONE

Step	*Chromatographic system*	*Steroid*	*μg*	*Total counts/min*	*Specific activity (counts/min per μmole)*
1	*Tert.*-butanol–isooctane–water	—	—	236 960	—
2	Toluene–isooctane–70% aq. methanol	B	90	188 100	723 140
3	Toluene–75% aq. methanol	B	72	158 760	760 184
4	Toluene–isooctane–70% aq. methanol	B-acetate	48	95 830	774 625
5	Light petroleum–benzene–80% aq. methanol	11-Dehydrocorticosterone acetate	38.6	73 805	738 050

Mean specific activity (Steps 3–5) = 757 619 ± 13 046 counts/min/μmole (± 1.72%)

dilution of the ^{14}C substance. The identification steps of 18-hydroxycorticosterone are shown in Table II.

Corticosterone (B): The corticosterone zone originating from the initial chromatogram was diluted with 100 μg of authentic carrier and carried through the steps indicated in Table III. The relative constancy of the specific activities between steps 4 and 5 (4.9%) gives a reasonable assurance, that the ^{14}C material contained no appreciable contamination with 17α-hydroxycorticosterone (Reichstein's substance S).

Cortisol and cortisone: Only very small amounts of cortisol could be isolated from the incubation medium. After dilution with 100 μg of carrier, a specific activity of 3890 counts/min/μmole was obtained. This specific activity remained constant after

chromatography before and after oxidation with sodium bismuthate (3850 and 3895 counts/min/μmole). However, the same procedure applied to the suspected [^{14}C]cortisone resulted in the isolation of androst-4-ene-3,11,17-trione devoid of significant amounts of radioactivity.

11-Deoxycorticosterone (DOC): This substance was present in the initial chromatogram near the solvent front. The radioactive substance was diluted with 200 μg of carrier, twice rechromatographed and finally transformed to its 21-acetoxy derivative. This product was further diluted with authentic 11-deoxycorticosterone-21-acetate (14.4 mg) and recrystallized twice from light petroleum–benzene. The three specific activities had a coefficient of variation of $\pm$2.1% (444, 418 and 433 counts/min per mg). This was interpreted as proof of identity between the ^{14}C material and the carrier steroid.

TABLE IV

THE CONVERSION OF [4-^{14}C]PROGESTERONE TO ADRENOCORTICOSTEROIDS BY DOMESTIC DUCK ADRENAL SLICES *in vitro*

Product of conversion as per cent of substrate added					
18OH B	*Corticosterone*	*Aldosterone*	*DOC*	*Cortisol*	*Progesterone*
15.33	11.43	6.85	0.38	0.05	7.49

The quantitative transformation of the substrate into the above mentioned compounds is shown in Table IV. As it can be seen, these components constitute some 41.5% of the initial activity. The initial crude solvent extract of the incubation medium yielded a recovery of 70.7% of the original activity of the substrate. After the first paper chromatography, the total radioactivity present was 59.7% of the [^{14}C]progesterone. The difference between the total activity of the identified compounds and that of the first radiochromatogram, representing some $2.99 \cdot 10^5$ counts per min, can be partly accounted for by inevitable losses, and in addition by a large number of small radioactive peaks which could not be identified. It has to be noted that the presence of 11-deoxy-18-hydroxycorticosterone was investigated, but the compound could not be demonstrated.

DISCUSSION

There is little doubt at present that lower classes of vertebrates secrete qualitatively the same type of corticosteroids as do mammals. However, it seems that quantitatively, 18-oxygenated steroids play a much more important role the lower we go in the evolutionary scale.

The principal corticosteroids derived from progesterone in the duck seem to be 18-hydroxycorticosterone, corticosterone and aldosterone. In this aspect, the duck adrenal resembles functionally the zona glomerulosa of beef adrenals. Very similar secretion patterns have been reported for adrenals of the bullfrog[5,6], lizard and grass snake[3] and pond turtle[22]. A comparison of these patterns is shown in Table V. In none of these animals was appreciable amount of 17-hydroxylase activity present. The data obtained in the domestic duck give rise to some interesting speculation. It has been demonstrated by PHILLIPS *et al.*[11,12] that the duck, similarly to marine

TABLE V

THE PER CENT TRANSFORMATION *in vitro* OF RADIOACTIVE PROGESTERONE TO ALDOSTERONE, 18-OH B AND CORTICOSTERONE BY ADRENALS OF DIFFERENT SPECIES OF VERTEBRATES

Species	*Label*	*Aldosterone*	*18-OH B*	*Corticosterone*	*Reference*
Rat	^{3}H	0.95	1.04	22.73	24
Beef (zona glomerulosa)	^{14}C	3.5	3.9	18.4	14
Domestic duck ♀	^{14}C	6.8	15.5	11.4	Present paper
Snake (*Natrix natrix*) ♂	^{3}H	1.9	5.5	13.9	3*
Snake (*Natrix natrix*) ♂	^{3}H	4.6	9.2	16.2	3*
Lizard (*Lacerta viridis*) ♀ + ♂	^{3}H	2.9	2.9	17.8	3*
Turtle (*Pseudemys*) ♀ + ♂	^{14}C	2.2	6.1	14.7	22
Frog (*Rana catesbiana*)	^{14}C	5.0	3.5	Not available	6

* These data are expressed as per cent transformation per 100 mg of tissue. The value represented as 18OH B refer to a substance not identified by the authors. However, according to its reported chromatographic behaviour, the material is most probably identical with 18OH B.

birds, can excrete a concentrated solution of NaCl from a pair of supraorbital nasal glands. This function of these glands is abolished following adrenalectomy; however the secretion from the nasal glands could be reestablished in these birds following injection of cortisol. PHILLIPS *et al.*[12] demonstrated that aldosterone is not essential for the normal functioning of these glands under salt loading. In view of this, an attractive hypothesis would be to postulate that 18-hydroxycorticosterone is a regulator of the function of supraorbital glands. There is very little known on the biological activity of 18-hydroxycorticosterone Recently, it has been established that in man its secretion always parallels that of aldosterone[23]. In the adrenalectomized rat, it has a weak sodium-retaining activity[23]. However, the biological activity of this compound has yet to be tested in lower vertebrates, especially in those which are equipped with extrarenal electrolyte-excreting mechanisms. The almost complete absence of 17-hydroxylated corticosteroids in the duck adrenals was a rather surprising fact. It is quite probable that the duck secretion pattern cannot be regarded as typical for the class of birds, but rather typical for the marine birds. DEROOS[1], working with cockerel adrenals, found that the three major corticoids secreted were corticosterone, aldosterone and cortisol in a ratio of 20:4:1. The almost total absence of 17-hydroxylation might have some connection with the aquatic origin of the duck.

One more point merits some discussion. In all incubations utilizing adrenals of lower vertebrates, progesterone was used as precursor. It is not even established with absolute certainty that in mammals progesterone is the sole precursor of 18-oxygenated steroids. The exploration of earlier precursors does merit some investigation, especially in lower vertebrates.

ACKNOWLEDGEMENTS

We are indebted to Dr. C. W. MURPHY of Ciba Company, Montreal and to Drs. R. NEHER AND J. SCHMIDLIN of CIBA, A.G. Basel, for gifts of synthetic *dl*-aldosterone and synthetic *dl*-18-hydroxycorticosterone. We wish to thank

Dr. F. G. PERON of the Worcester Foundation of Experimental Biology, Shrewsbury, Mass., for his generous gift of biosynthetic 18-hydroxy-11-deoxy[17α-^{3}H]corticosterone. The invaluable technical assistance of Mrs. A. LIEBERT is gratefully acknowledged. This study was supported by grants from the Medical Research Council of Canada, the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service (A-4327) and the Ciba Company, Montreal.

REFERENCES

1 R. DEROOS, *Endocrinology*, 67 (1960) 719.
2 R. DEROOS, *Gen. Comp. Endocrinol.*, 1 (1961) 494.
3 F. G. PHILLIPS, I. CHESTER-JONES AND D. BELLAMY, *J. Endocrinol.*, 25 (1962) 233.
4 H. CARSTENSEN, A. C. F. BURGER AND C. H. LI, *Gen. Comp. Endocrinol.*, 1 (1961) 37.
5 S. ULICK AND S. SOLOMON, *J. Am. Chem. Soc.*, 82 (1960) 249.
6 S. ULICK AND K. KUSCH, *J. Am. Chem. Soc.*, 82 (1960) 6421.
7 J. CRABBÉ, *Endocrinology*, 69 (1961) 673.
8 J. G. PHILLIPS AND P. J. MULROW, *Proc. Soc. Exptl. Biol. Med.*, 101 (1959) 262.
9 J. NANDI AND H. A. BERN, *Endocrinology*, 66 (1960) 295.
10 W. N. HOLMES, J. G. PHILLIPS AND D. G. BUTLER, *Endocrinology*, 69 (1961) 483.
11 J. G. PHILLIPS, W. N. HOLMES AND D. G. BUTLER, *Endocrinology*, 69 (1961) 958.
12 J. G. PHILLIPS AND D. BELLAMY, *J. Endocrinol.*, 24 (1962) vi.
13 W. N. UMBREIT, R. H. BURRIS AND J. F. STAUFFER, *Manometric Techniques and Tissue Metabolism*, Burgess Publishing Co. Minneapolis, Minn., 1957.
14 T. SANDOR AND A. LANTHIER, *Acta Endocrinol.*, 39 (1962) 87.
15 T. SANDOR AND A. LANTHIER, *Acta Endocrinol.*, 42 (1963) 355.
16 W. J. NOWACZYNSKI, M. GOLDNER AND J. GENEST, *J. Clin. Lab. Med.*, 45 (1955) 818.
17 D. BERLINER AND H. A. SALHANICK, *Anal. Chem.*, 28 (1956) 1608.
18 S. A. SIMPSON, F. J. TAIT, A. WETTSTEIN, R. NEHER, J. VON EUW, O. SCHINDLER AND T. REICHSTEIN, *Helv. Chim. Acta*, 37 (1954) 1200.
19 G. I. POOS, G. E. ARTH, R. E. BEYLER AND L. H. SARETT, *J. Am. Chem. Soc.*, 75 (1953) 422.
20 C. J. W. BROOKS AND J. L. NORYMBERSKI, *Biochem. J.*, 55 (1953) 371.
21 E. A. HAM, R. E. HARMAN, N. G. BRINK AND L. H. SARETT, *J. Am. Chem. Soc.*, 77 (1955) 1637.
22 T. SANDOR AND A. LANTHIER, *Federation Proc.*, 22 (1963) 655.
23 S. ULICK AND K. KUSCH-VETTER, *J. Biol. Chem.*, 237 (1962) 3364.
24 F. G. PÉRON, *Endocrinology*, 70 (1962) 386.