Corticosteroidogenesis in the toad *Bufo arenarum* H: evidence for a precursor role for an aldosterone 3 β -hydroxy-5-ene analogue (3 β ,11 β , 21-trihydroxy-20-oxo-5-pregnen-18-al)*

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A material isolated following pregnenolone incubations with toad (*Bufo arenarum*) inter-renal tissue at 28 °C has been identified as a 3 β -hydroxy-5-ene analogue of aldosterone (3 β ,11 β ,21-trihydroxy-20-oxo-5-pregnen-18-al). The initial identification was made by enzymic and m.s. methods, and structural confirmation was achieved through comparison with

chemically synthesized authentic material. The relative efficacy of corticosterone, 18-hydroxycorticosterone and the 3 β -hydroxy-5-ene aldosterone analogue as aldosterone precursors was evaluated. In the *in vitro* situation studied, the 3 β -hydroxy-5-ene steroid was by far the best precursor.

INTRODUCTION

In a previous publication [1], we reported that in 28 °C incubations of inter-renal tissue of *Bufo arenarum* (the predominant amphibian in the southern region of South America), pregnenolone was largely converted into aldosterone and an unknown 'Compound N'. Corticosterone was less efficiently utilized. These results suggested an atypical aldosterone synthetic route which may have utilized a '3 β -hydroxy-5-ene' pathway, rather than the classical 3-oxo-4-ene pathway. By contrast, in the rat, Compound N was not obtained as a product, either in 28 or 37 °C incubations (N. R. Ceballos, E. N. Cozza and C. P. Lantos, unpublished work).

In the present study we characterize the unknown Compound N and evaluate its possible role as an aldosterone precursor.

MATERIALS AND METHODS

Steroids and enzymes

The steroid 3 β ,11 β ,21-trihydroxy-20-oxo-5-pregnen-18-al (the 3 β -hydroxy-5-ene analogue of aldosterone) principally exists in mono- or bi-cyclic forms 11 β ,18-epoxy,3 β ,18,21-trihydroxy-5-pregnen-2-one and 11 β ,18;18,20-diepoxy-5-pregnen-3 β ,20,21-triol. It was synthesized as described by Harnik et al. [2]. Corticosterone and pregnenolone were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 18-hydroxycorticosterone was obtained from Makor Chemicals (Jerusalem, Israel); [³H]corticosterone (40 Ci/mmol) was from NEN (Boston, MA, U.S.A.); [³H]pregnenolone (19 Ci/mmol) and [¹⁴C]aldosterone (57 mCi/mmol) were also from NEN. Hydroxysteroid dehydrogenase was obtained from Sigma Chemical Co. (grade II from *Pseudomonas testosteroni*).

Tissue preparations

Inter-renal tissue was obtained from toads (*Bufo arenarum*

Hensel) captured near Buenos Aires during the spring or summer. The glands were excised and homogenized with buffer containing potassium phosphate (20 mM), MgCl₂ (4 mM), glucose (9 mM) and sucrose (0.25 M), at pH 7.4 (250 mg of tissue/ml) as described by Psychoyos et al. [3].

Unlabelled and ³H-labelled Compound N and 18-hydroxycorticosterone were obtained by incubation of tissue slices with pregnenolone according to the following method [1]. Pregnenolone (20 μ M) and [³H]pregnenolone (2 μ Ci) were incubated for 3 h with 60 mg of tissue in Krebs–Ringer bicarbonate buffer containing glucose. The steroid products were extracted with dichloromethane and purified by paper chromatography employing a toluene/propylene glycol system [4]. Final separation of the steroids of interest (remaining at origin) was obtained by development in the B5 Bush system [5]. The biologically synthesized Compound N (*R_f* 0.21) and 18-hydroxycorticosterone (*R_f* 0.13) were located and eluted, and radioactivity was determined by liquid-scintillation counting. Compound N was further purified by h.p.l.c. using a Waters instrument, μ Bondapak C₁₈ columns, and methanol/water (3:2, v/v) as elution solvent. The purified Compound N was subjected to m.s. characterization. Compound N, corticosterone and 18-hydroxycorticosterone were used as precursors to study aldosterone biosynthesis.

Aldosterone biosynthesis

Compound N, corticosterone and 18-hydroxycorticosterone were incubated with homogenates of toad inter-renal tissue. Enzymes corresponding to 50 mg of tissue were employed for corticosterone and 18-hydroxycorticosterone and 30 mg of tissue for Compound N. The incubations were carried out at 28 °C for 30 min in 5 ml beakers with an incubation volume of 1 ml. Substrate concentration was 10 μ M, and substrate specific radioactivity was 72 mCi/mmol. These conditions were established by preliminary experiments as corresponding to the linear zones of time and enzyme curves. The following compounds were used in

Abbreviations used: (MO-)TMS, (methyloxime)trimethylsilyl ethers; M⁺, molecular ion.

* The present paper is the second of a series of papers on this topic. Paper 1 is [1].

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various different experiments: malate (5 mM); NAD⁺, NADP⁺, NADH and NADPH (0.5 mM).

Recovery of aldosterone

The products of inter-renal-tissue incubation were extracted with dichloromethane and purified by the method of Kliman and Peterson [6] as modified by Ceballos et al. [1]. Briefly, [¹⁴C]aldosterone and the dichloromethane extract residue were co-chromatographed in the Bush B5 paper-chromatographic system [5]. The dry paper (Whatman no. 1) to which the sample had been applied was hung in the chromatography tank in the presence of the solvent system (benzene/methanol/water, 2:1:1, by vol.) and was allowed to equilibrate for 2 h. The paper preferentially absorbs the polar stationary phase (enriched in methanol and water), and the chromatogram was then developed with the less-polar phase. It was found that a co-eluted band of R_f 0.25 included [¹⁴C]aldosterone and aldosterone-like ³H-labelled material. These were eluted from the chromatogram with methanol, dried under nitrogen and acetylated with 200 μ l of acetic anhydride and 200 μ l of anhydrous pyridine. This material was then rechromatographed in the paper-chromatographic system cyclohexane/dioxan/methanol/water (4:4:2:1, by vol.) [5]. The acetate after dilution was oxidized with 0.5% CrO₃ in acetic acid for 15 min. The aldolactone was chromatographed sequentially in the former system and in cyclohexane/benzene/methanol/water (4:4:2:1, by vol.). The amount of [³H]aldosterone produced was quantified by taking into account losses of added ¹⁴C tracer after the last purification step. Homogeneity was achieved by determining the ³H/¹⁴C ratio of the initial isolate and chemically modified aldosterone and ascertaining that it was constant.

3 β -Hydroxysteroid dehydrogenase incubation

Incubation was carried out in pyrophosphate buffer in the presence of NAD⁺ at pH 8.9 [7] with 0.06 unit of β -hydroxysteroid dehydrogenase and 0.4 mM NAD⁺ in a final volume of 3 ml. The substrates utilized were pregnenolone, Compound N, the 3 β -hydroxy analogue of aldosterone, corticosterone and aldosterone. The two latter were used as controls, as they should not be transformed. At the same time, their use demonstrates that aldosterone is not further metabolized by the enzyme or the enzyme source after adding NAD⁺. The steroids were added to the aqueous incubation mixture by dissolving them in 50 μ l of propylene glycol. Conditions were those suggested by Sigma to transform a 3 β -hydroxysteroid into a 3-ketone. The metabolites were extracted out and purified by paper chromatography in the following systems: metabolites of aldosterone, Compound N and the aldosterone analogue, in the Bush B5 system; metabolites of corticosterone in toluene/propylene glycol and metabolites of pregnenolone by t.l.c. (silica-gel GF₂₅₄ on aluminium; Merck Chemical Co.) in benzene/acetone (4:1, v/v).

M.s.

Steroids were analysed according to methods previously described [8]. In brief, trimethylsilyl ethers (TMS) and methyloxime trimethylsilyl ethers (MO-TMS) were prepared as follows. TMS derivatives were prepared in glass test tubes using 100 μ l of bistrimethylsilyl trifluoroacetamide containing 1% trimethylsilylimidazole. After heating at 60 °C for 1 h, the samples were

directly injected into the mass spectrometer. The MO derivatives were prepared by allowing the samples to remain overnight in 100 μ l of a solution of methoxyamine hydrochloride (2%, v/v, in pyridine). The samples were dried under nitrogen, 100 μ l of trimethylchlorosilane was added and silylation allowed to proceed for 1 h at 100 °C. The MO-TMS derivatives were purified by Lipidex chromatography to remove excess reagents [9]. Cyclohexane was used as the final solution for introduction into the g.c.-m.s. instrument (Hewlett-Packard 5970A). A 15-metre DB-1 non-polar fused silica column (OV-1 type) was used for the steroid separations. The samples were introduced by syringe injection with cold-trapping at 60 °C. After 3 min the temperature was rapidly increased to the 220 °C starting temperature of the program. Thereafter the temperature was increased linearly by 3°/min to the final temperature of 330 °C. Mass spectra were obtained by continuous repetitive scanning over the 100–700 mass range.

RESULTS

Characterization of Compound N

Since Compound N was produced from pregnenolone, the presence of a 3 β -hydroxy-5-ene steroid was always a likely possibility. For this reason we incubated it with 3 β -hydroxysteroid dehydrogenase. Both it and pregnenolone were converted into less polar products, indicating the presence of a 3 β -hydroxy group.

Early in these studies it was considered that Compound N may be a 3 β -hydroxy-5-ene analogue of aldosterone, i.e. 3 β ,11 β ,21-trihydroxy-20-oxo-5-pregnen-18-al. One of us synthesized this reference material some years ago [2] and showed that it existed in the monocyclic and bicyclic forms illustrated below:

As a solid it was principally in bicyclic form, while n.m.r. showed that, in [²H]chloroform solution, the amount of each present was

Table 1 G.c. retention times (t_R , min) and methylene unit values (MU) for Compound N and 3 β ,11 β ,21-trihydroxy-20-oxo-pregnen-18-al as TMS and MO-TMS derivatives

Derivative	Parameter	Peak 1	Peak 2	Peak 3
TMS	t_R			
	Compound N	25.99	26.79	
	Standard	25.94	26.76	
	MU			
MO-TMS	t_R			
	Compound N	25.14	26.10	26.97
	Standard	25.12	26.09	26.98
	MU			
	Compound N	30.90	31.25	31.65
	Standard	30.88	31.30	31.65

Figure 1 Mass spectra of the TMS derivative of Compound N

Two peaks were given with the minor peak giving Spectrum A and a major peak giving Spectrum B. In each case the molecular ions (M^+) were unseen at m/z 578. Two peaks with the same retention times and mass spectra were obtained for the TMS derivative of reference $3\beta,11\beta,21$ -trihydroxy-20-oxo-5-pregnen-18-al.

approximately equal [2]. Thus it was not unexpected to find that this synthesized compound and the material we were anticipating to prove identical (Compound N), as TMS derivatives gave two peaks when analysed by g.c.-m.s. The absolute retention times and Kováts retention indices (methylene units) of both peaks are given in Table 1. Clearly, the synthesized and biologically isolated material have the same retention times. The mass spectra of the peaks of Compound N TMS derivatives are illustrated in Figure 1. The corresponding spectra of the synthesized material were identical. The second, and major component had spectrum B indicating a molecular ion (M^+) at 578 and major fragmentations resulting from loss of the C-21 primary hydroxy group ($M-103$) and incremental forms of trimethylsilanol (-90). The ion at m/z 295 is the core after removal of all the derivatized moieties, and the ion at m/z 239 the basic ring structure remaining after loss of C-20 with oxygen and C-18 with oxygen (m/z 295-46). This spectrum is similar to that previously published by us for tetrahydroaldosterone, the only major difference being that, for

Figure 2 Mass spectra of the MO-TMS derivative of Compound N

Three peaks were found: a minor initial peak giving Spectrum A and two major peaks giving identical spectra (Spectrum B). The two major peaks were *syn*- and *anti*-forms of the MO derivative. M^+ (unseen) is at m/z 607, and the base peak is at m/z 504. Three peaks with the same retention times and mass spectra were found for reference $3\beta,11\beta,21$ -trihydroxy-20-oxo-5-pregnen-18-al.

the latter compound, the fragment ions were 2 mass units higher [10]. The first chromatographic peak was missing M^+ at m/z 578, but had essentially the same ions but with very different relative abundances. If one of these peaks represents the bicyclic form and one the monocyclic, how do we know which is which? We note that the g.c. peak shape of the second peak was much broader, which we interpreted as being due to presence of an unprotected polar group in the molecule, and only the monocyclic form has one: the C-20 carbonyl group. Thus we believe the major form of the TMS derivative is the monocyclic form. Data produced from the TMS derivative alone showed that Compound N and synthesized steroids were identical, since they only gave two peaks and both gave identical spectra.

As MO-TMS derivatives, both Compound N and the synthesized material gave three chromatographic peaks (Figure 2). The retention times of products from the authentic and biologically synthesized compound were considered identical. Peaks 2 and 3 were of equal size and presumably represent the *syn*- and

Table 2 $^3\text{H}/^{14}\text{C}$ ratios during purification of aldosterone

Abbreviation: Aldo-like, aldosterone-like material. For cofactor concentrations, see the Materials and methods section. These results represent the means for four individual experiments.

Substrate and cofactors	$^3\text{H}/^{14}\text{C}$ ratio				
	Initial	Aldo-like	Aldosterone acetate	First lactone	Second lactone
Corticosterone					
NADP ⁺ + malate	72.31	7.45	2.10	1.60	1.62
NADPH	70.35	6.38	1.97	1.36	1.37
Compound N					
NAD ⁺	38.05	8.61	8.39	8.32	8.64
NAD ⁺	25.34	6.72	6.87	6.90	6.68

Table 3 Incubation of corticosterone, 18-hydroxycorticosterone and Compound N with inter-renal-tissue homogenate

Abbreviation: N.D., non-detectable. Ten toads were used for each experiment. Values are means $\pm$ S.E.M. The cofactor concentrations are given in the Materials and methods section.

Substrate	Cofactor(s)	Conversion into aldosterone (pmol of aldosterone produced/min per mg of tissue)
Corticosterone	NADP ⁺ + malate	0.15 $\pm$ 0.03
	NADPH	0.13 $\pm$ 0.03
Synthetic 18-Hydroxycorticosterone	NADP ⁺ + malate	N.D.
	NADPH	N.D.
Toad-18-Hydroxycorticosterone	NADP ⁺ + malate	N.D.
	NADPH	N.D.
Compound N	Alone	0.94 $\pm$ 0.11
	NADP ⁺ + malate	0.97 $\pm$ 0.11
	NADP ⁺	0.99 $\pm$ 0.10
	NADPH	0.91 $\pm$ 0.13
	NADH	0.99 $\pm$ 0.11
	NAD ⁺	2.74 $\pm$ 0.24

anti-forms of the C-20 oxime. Their spectra were identical, and no differences were noted between the spectra for Compound N and the synthesized standard. In the mass spectra (Figure 2, Spectrum A, here illustrated for Compound N), the highest mass ion was at m/z 592, which was interpreted as $M - 15$ of an absent M^+ (m/z 607). The base peak was at m/z 504 ($M - 103$), the loss of 103 mass units being once again diagnostic of the presence of a primary hydroxy group (C-21). These spectra were also essentially the same as those previously published for tetra-hydroaldosterone [10], the relevant ions of the latter compound being 2 mass units higher. The mass spectrum of the first and minor component of Compound N and the synthesized standard (as MO-TMS derivative) is illustrated in Spectrum B. The ion at m/z 592 suggests also that the M^+ was at 607, but the spectrum was very different from that of the major components. Clearly, this represents a different or rearranged form of the compound also containing three TMS groups and one oxime. A structure

that must be considered is one where a free C-20 carbonyl is maintained while the C-18 carbonyl is oximated. In spite of the fact that synthesized standard and compound N had a 3β -hydroxy-5-ene group, neither gave a major ion at m/z 129, an ion often dominant in TMS and MO-TMS derivatives of this type of steroid.

The evidence presented above shows that the purified sample of Compound N contains only the isomeric forms of the 3β -hydroxy-5-ene analogue of aldosterone, so this material may be deemed homogeneous.

Efficiency of different steroids as aldosterone precursors

It was necessary to confirm that aldosterone was indeed formed during incubations. Table 2 shows the $^3\text{H}/^{14}\text{C}$ ratios of isolated

Scheme 1 Summary of aldosterone biosynthesis in *Bufo arenarum*

The following intermediates in the conventional (3-oxo-4-ene) pathway are indicated: 1, pregnenolone; 2, progesterone; 3, deoxycorticosterone; 4, corticosterone; 5, 18-hydroxycorticosterone and 6, aldosterone. Compound N ($3\beta,11\beta,21\beta$ -trihydroxy-20-oxo-5-pregnen-18-al) would be the final intermediate in a corresponding 3β -hydroxy-5-ene pathway.

aldosterone and its subsequently produced acetate and lactone derivatives [6], showing achievement of constant ratio, verifying aldosterone production from Compound N and corticosterone. The standard 3 β -hydroxy-5-ene analogue of aldosterone yielded under the same conditions a u.v.-absorbing compound with the same chromatographic mobilities as aldosterone. The results show that Compound N in the presence of NAD⁺ as cofactor metabolizes to an 'aldosterone-like' fraction containing only aldosterone, while corticosterone in the presence of NADPH metabolizes to an aldosterone-like fraction containing large amounts of other materials besides aldosterone.

Table 3 shows results obtained during incubations of corticosterone, 18-hydroxycorticosterone and Compound N. 18-Hydroxycorticosterone was essentially not converted into aldosterone, but corticosterone and Compound N did give rise to the hormone, although the latter was a much more efficient precursor, particularly when NAD⁺ was used as cofactor. It was not surprising that 18-hydroxycorticosterone was a poor precursor, as this is also the case in the rat where the 4-ene pathway is dominant. One cause could be the 18,20-hemiacetal nature of the added compound, which prevents its interaction with the enzymes responsible for oxidation. Potential factors inhibiting the conversion of exogenous 18-hydroxycorticosterone have been discussed by Lantos and co-workers [11].

DISCUSSION

Preliminary evidence for a possible 3 β -hydroxy-5-ene pathway to aldosterone at 28 °C obtained in our previous investigation [1] has been confirmed in the present study by the characterization of an aldosterone precursor previously designated Compound N as the 3 β -hydroxy-5-ene analogue of the mineralocorticoid. The greater efficiency *in vitro* of this steroid as an aldosterone precursor also provides substantial evidence for its importance. The results demonstrated that the conversion was a two-step reaction catalysed by 3 β -hydroxy steroid dehydrogenase 4,5-isomerase requiring NAD⁺ as cofactor. The proposed position of the analogue in the biosynthetic pathway to aldosterone in *Bufo arenarum* is illustrated in Scheme 1.

The results of present and former studies suggest that progesterone, deoxycorticosterone and corticosterone can be bypassed by the toad's inter-renal tissue during aldosterone biosynthesis. This previously unreported feature in the Animal Kingdom may be explained by the toad's osmotic requirements. Its fight against dehydration, especially during metamorphosis from an aqueous tadpole to a terrestrial adult, singles out aldosterone as a main corticoid in this and other amphibian species [12,13]. A 3 β -hydroxy-5-ene pathway to the highly specialized and structurally unique mineralocorticoid would

avoid the formation of hormones of different activities which are superfluous or even harmful. It should be emphasized that the 3 β -hydroxy-5-ene aldosterone analogue is a sodium-retaining steroid in its own right, although a weaker one than aldosterone [14].

The incubation temperature at which the 3 β -hydroxy-5-ene pathway predominates is 28 °C [1]. This is usually considered as the normal physiological temperature for amphibians, although *Bufo*'s habitat extends over multiclimate zones from the tropics to cold climate, occasionally reaching freezing temperatures in winter. The reason why at 37 °C the 3-oxo-4-ene pathway and with it the biosynthesis of corticosterone and related steroids becomes important is still intriguing. These results and speculations must be treated with caution, since *in vitro* studies do not necessarily reflect the *in vivo* situation. These studies should be supplemented by determination of steroid levels in plasma of toads kept at different temperatures, although it must also be borne in mind that precursor type steroids (e.g. Compound N) do not necessarily find their way into the circulation. The data do suggest that extremely high temperatures, in spite of *Bufo*'s large habitational range, could be harmful, and that under these conditions this amphibian would require a certain concentration of glucocorticoids to face stressful exposures.

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REFERENCES

- 1 Ceballos, N. R., Cozza, E. N. and Lantos, C. P. (1983) *Gen. Comp. Endocrinol.* **51**, 138–147
- 2 Harnik, M., Kashman, Y., Aharonowitz, Y. and Morris, D. J. (1985) *J. Steroid Biochem.* **23**, 207–218
- 3 Psychoyos, S., Tallan, H. H. and Greengard, P. (1966) *J. Biol. Chem.* **241**, 2949–2956
- 4 Zaffaroni, A. and Burton, R. B. (1951) *J. Biol. Chem.* **193**, 749–767
- 5 Bush, I. C. and Willoughby, M. (1957) *Biochem. J.* **67**, 689–700
- 6 Kliman, B. and Peterson, R. E. (1960) *J. Biol. Chem.* **235**, 1639–1648
- 7 Marcus, P. I. and Talalay, P. (1956) *J. Biol. Chem.* **218**, 661–674
- 8 Shackleton, C. H. L. (1986) *J. Chromatogr.* **379**, 91–156
- 9 Axelson, M. and Sjövall, J. (1974) *J. Steroid Biochem.* **5**, 733–738
- 10 Honour, J. W. and Shackleton, C. H. L. (1977) *J. Steroid Biochem.* **8**, 299–305
- 11 Lantos, C. P., Damasco, M. C., Aragonés, A., Ceballos, N. R., Burton, G. and Cozza, E. N. (1987) *J. Steroid Biochem.* **27**, 791–800
- 12 Carstensen, H., Burgers, A. C. J. and Li, C. H. (1959) *J. Am. Chem. Soc.* **81**, 4109–4110
- 13 Leboulenger, F., Delarue, C., Jegou, S., Netchitailo, P., Leroux, P., Tonon, M. C. and Vaudry, H. (1980) *Gen. Comp. Endocrinol.* **42**, 389–400
- 14 Morris, D. J., Brem, A. S., Saccoccio, N. A., Pacholski, M. and Harnik, M. (1986) *Endocrinology (Baltimore)* **118**, 2505–2509