Hydroxylation of Deoxycorticosterone at the 6β, 11α, 15α, 15β, and 16α Positions by Surviving Liver Slices of the Bullfrog*

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ABSTRACT: 6β-, 11α-, 15α-, 15β-, and 16α-Hydroxydeoxycorticosterone were isolated following the aerobic incubation of deoxycorticosterone with surviving liver slices of the bullfrog. These metabolites were identified by infrared spectroscopy and further characterized by paper chromatographic methods. The results were compared with those hydroxylations observed where mammalian tissues were employed.

In an earlier comparative study of the 7α-hydroxylation of deoxycorticosterone (compound I) by surviving liver slices of various species (Schneider, 1962), it was noted that, while this reaction occurred only in rodent tissue, certain nonrodent tissue introduced hydroxyl groups into several positions other than C-7. The paper chromatographic pattern obtained in the case of the bullfrog (*Rana catesbieana*) was particularly complex and, since this species is readily available, several large-scale incubations were carried out with the object of recovering the metabolites in pure form. These were identified as 6β-, 11α-, 15α-, 15β-, and 16α-hydroxydeoxycorticosterone. It is the purpose of this paper to detail their isolation and identification.

Methods

The incubation, extraction, and paper chromatographic procedures followed those described earlier (Schneider, 1962). The reference steroids were supplied by Dr. Robert Neher. Extinction coefficients were obtained in methanol solution using a Zeiss PMQ II spectrophotometer. The infrared spectra of both the reference and isolated steroids were determined as KBr pellets by the use of a Beckman IR-9 instrument for the former and a Perkin-Elmer Model 21 instrument for the latter. The use of two different instruments did not lead to difficulties in comparing the spectra. The bullfrogs used in these incubations were obtained from commercial sources in Wisconsin and New Jersey.

Results

In the representative experiment here described, 500 mg of free compound I was incubated (3 hours at 38°, gas-phase oxygen) with liver slices from 200 large male or female bullfrogs, corresponding to a steroid-to-tissue ratio of about 1:750. The crude neutral extract was applied as 16-cm "lines" on twenty-four 19 × 60-cm sheets of Whatman No. 1 paper impregnated with formamide and chromatographed for 30 hours using system 1.[1] Scanning with a lamp emitting maximally at approximately 253 mμ showed the presence of three well-separated bands (designated 1, 2, and 3) which were marked, cut out, and eluted as described (Schneider, 1962).

Band 1 (the polar or slowest-moving fraction) was applied as 16-cm lines on twenty 19 × 60-cm sheets, equilibrated overnight, and chromatographed for 8 hours using system 2. This divided the applied band into a smaller, polar fraction (labeled 1-P) and a larger, mobile component (1-M). Band 2 (the fraction of intermediate mobility) was applied as 16-cm lines on twenty-six 19 × 60-cm sheets, equilibrated overnight, and chromatographed for 8 hours using system 3. Scanning showed good separation into a smaller, polar zone (2-P) and a larger, mobile component (2-M). Rechromatography of small aliquots of band 3 (the mobile component) in several systems showed that it consisted of a single steroid. As a preliminary to recovery in crystalline form, the crude fraction was applied as wide bands on four 19 × 60-cm sheets and chromatographed for 48 hours using system 4 as the mobile phase. The zone of interest was designated R-3.

Identification of the pure metabolites was based primarily on comparisons of their infrared spectra with those of the reference steroids. This information, together with the extinction coefficients of the isolated steroids and the melting points of suitable mixtures, is cited where pertinent. In addition, the paper chromatographic mobilities of the isolated and reference steroids were compared in six systems. These results, which were in agreement with the identifications based

* From the Department of Medicine, Jefferson Medical College, Philadelphia, Pa. *Received December 8, 1964.* This work was supported wholly by a research grant (AM 01255) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service, and was presented in part at the June 1962 meeting of the Endocrine Society.

[1] Systems 1, 2, 3, 4, 6, and 7 appear in Table I, system 5 appears under Results, and system 8 is given in Table II.

TABLE I: $R_{s(corticosterone)}$ Values[a] for Isolated Deoxycorticosterone Metabolites in Various Paper Chromatographic Systems.

No. and Site of Hydroxylation	System[b]					
	1	2	3	4	6	7
II (15α)	0.23 (0.19)	0.37	0.45	0.17	0.24 (0.23)	0.43 (0.45)
III (16α)	0.19	0.68	0.74	0.14	0.21	0.41 (0.41)
IV (15β)	0.31	0.46	0.48	0.23	0.40	0.53 (0.45)
V (11α)	0.35	0.62	0.74	0.28	0.38	0.56 (0.56)
VI (6β)	0.50 (0.50)	[c]	[c]	0.44 (0.49)	0.59 (0.59)	0.67 (0.73)

[a] Since the R_s values for the simultaneously chromatographed metabolites and corresponding reference steroids agreed in all cases within the limits of error of the method, only the values for the former are recorded. The values in parentheses are taken from the extensive compilations of Neher (1959, 1964). [b] System 1 = benzene saturated with formamide. System 2 = toluene, 20; isooctane, 130; *t*-butyl alcohol, 50; water, 150 ml. System 3 = isooctane, 150; *t*-butyl alcohol, 70; water, 130 ml. System 4 = benzene-cyclohexane, 1:1 (v/v), saturated with formamide. System 6 = toluene saturated with propylene glycol. System 7 = benzene-chloroform, 1:1 (v/v), saturated with formamide. Adequate movement of the more polar steroids required extensive overrunning with some systems. The steroids were detected on the dried chromatograms by ultraviolet-light scanning followed by application of the blue tetrazolium reagent (Neher, 1959). [c] Denotes streaking.

on infrared spectroscopy, are summarized in terms of $R_{s(corticosterone)}$ values in Table I. The structural formulas of the substrates and metabolites appear in Figure 1.

Recovery of 4-Pregnene-15α,21-diol-3,20-dione (15α-Hydroxydeoxycorticosterone, Compound II). Subfraction 1-P (11.2 mg) gave, from acetone-*n*-hexane and from acetone, 5.4 mg of colorless needles (λ_{max} = 241 mμ, ε = 15,600). Since the melting points of the isolated metabolites II and IV were not sharp (see also Meystre *et al.*, 1958), little value was attached to the melting point of compound II admixed with authentic 15α-hydroxydeoxycorticosterone[2] as an index of identity. The constitution of compound II was established on the basis of its infrared spectrum which corresponded closely with that obtained from authentic 15α-hydroxydeoxycorticosterone. Meystre *et al.* (1958) prepared 15α-hydroxydeoxycorticosterone ($\lambda_{max}^{ethanol}$ = 242 mμ, ε = 16,950) by incubating I with the fungus *Gibberella baccata*.

Recovery of 4-Pregnene-16α,21-diol-3,20-dione (16α-Hydroxydeoxycorticosterone, Compound III). The 1-M residue (13.8 mg) yielded, from acetone-*n*-hexane and from acetone, 10.2 mg of colorless needles having the following constants: mp 199–201° (after some softening from around 180°); λ_{max} = 241 mμ, ε = 16,700. The melting point of the isolated metabolite was unaltered on mixing with the reference sample of 16α-hydroxydeoxycorticosterone, which melted at 198–202°. The infrared spectra of the two were identical. 16α-Hydroxydeoxycorticosterone (mp 203–205°; $\lambda_{max}^{ethanol}$ = 241 mμ, ε = 16,300) was prepared by Vischer *et al.* (1954) by incubating compound I with a *Streptomyces* species and in the same fashion by Fried *et al.* (1955) using the bacterium *Streptomyces roseochromogenus*. Hirschmann *et al.* (1953) described the preparation of the diacetate of compound III by chemical means.

Recovery of 4-Pregnene-15β,21-diol-3,20-dione (15β-Hydroxydeoxycorticosterone, Compound IV). Subfraction 2-P (16.3 mg) was twice crystallized from methanol, furnishing 11 mg of well-formed cubes; λ_{max} = 241 mμ, ε = 16,700. For a time the paper chromatographic data appeared to offer little assistance in identifying this metabolite, but eventually it was noted that it resisted complete acetylation when treated with acetic anhydride and pyridine at room temperature. This property, unique among the metabolites here described, was found to be characteristic of 15β-hydroxyprogesterone (and presumably of compound IV) by Fried *et al.* (1955) who attributed it to the hindered, quasi-axial orientation of the 15β-hydroxyl group. The infrared spectra of pure compound IV and of authentic 15β-hydroxydeoxycorticosterone were then found to be identical. Meystre *et al.* (1958) prepared 15β-hydroxydeoxycorticosterone ($\lambda_{max}^{ethanol}$ = 242 mμ, ε =

[2] The indicated configurations at C-15 of the reference samples of 15α- and 15β-hydroxydeoxycorticosterone conform to the assignments of Djerassi *et al.* (1955) as later acknowledged by Wettstein (1955).

16,700) by incubating compound I with the fungus *Lenzites abietina*.

While the metabolites epimeric at C-15 are readily separated using system 1 with a running time of around 24 hours, another system was developed (toluene, 50; isooctane, 100; *t*-butyl alcohol, 30; water, 170 ml [system 5; its use is illustrated in Table II]) which separates the epimers by 75 mm in a running time of 7 hours.

TABLE II: ΔR_{M_9} Values[a] of Hydroxyl Groups.

No.	Identity	System[b] 3	5	7	8
1	6β-OH-I (a)[c]			1.02	1.13
2	7α-OH-I (a)	1.01	1.42	1.23	1.39
3	7β-OH-I (e)	1.11	1.48	1.16	1.45
4	11α-OH-I (e)	0.83	1.48	1.16	1.49
5	11β-OH-I (a)	0.54	0.88	0.74	0.88
6	15α-OH-I (quasi-e)	1.18	1.75	1.35	1.64
7	15β-OH-I (quasi-a)	1.18	1.59	1.24	1.43
8	16α-OH-I	0.88	1.51	1.35	1.54
9	17α-OH-I (a)	0.30	0.65	0.76	0.74

[a] All values are positive. [b] System 8 = toluene, 200; methanol, 130; water, 70 ml. Other systems correspond to that in text (system 5) or in Table I (systems 3 and 7). [c] 6β-Hydroxydeoxycorticosterone (no. 1), and so forth. The letter in parentheses indicates the configuration of the introduced hydroxyl group in terms of the axial (a) or equatorial (e) designation of Barton and Crookson (1956).

Recovery of 4-Pregnene-11α,21-diol-3,20-dione (11α-Hydroxydeoxycorticosterone, Compound V). Subfraction 2-M (30.4 mg) gave, from acetone-*n*-hexane, 22 mg of colorless needles, mp 154–156°; λ_{max} = 242 mμ, ε = 16,500. On admixture with the reference sample of 11α-hydroxydeoxycorticosterone (mp 153–155°), the melting point was unchanged. The infrared spectra of the isolated and reference steroids were identical. 11α-Hydroxydeoxycorticosterone (11-epicorticosterone) has been prepared microbiologically by Fried *et al.* (1952), by Kahnt *et al.* (1952), and by Eppstein *et al.* (1953).

Recovery of 4-Pregnene-6β,21-diol-3,20-dione (6β-Hydroxydeoxycorticosterone, Compound VI). Subfraction R-3 (26 mg) was crystallized from acetone-*n*-hexane and from methanol to give 16 mg of needles. Constants: mp 193–195°; λ_{max} = 237 mμ, ε = 13,100. The melting point of the metabolite was unaltered on admixture with the reference sample of 6β-hydroxydeoxycorticosterone which melted at 195–197°. Their infrared spectra were identical. 6β-Hydroxydeoxycorticosterone (mp 190–192°; $\lambda_{max}^{ethanol}$ = 235 mμ, ε = 13,730) was prepared chemically by Herzig and Ehrenstein (1951) and microbiologically (mp 198–202°; ε = 13,700) by Eppstein *et al.* (1953).

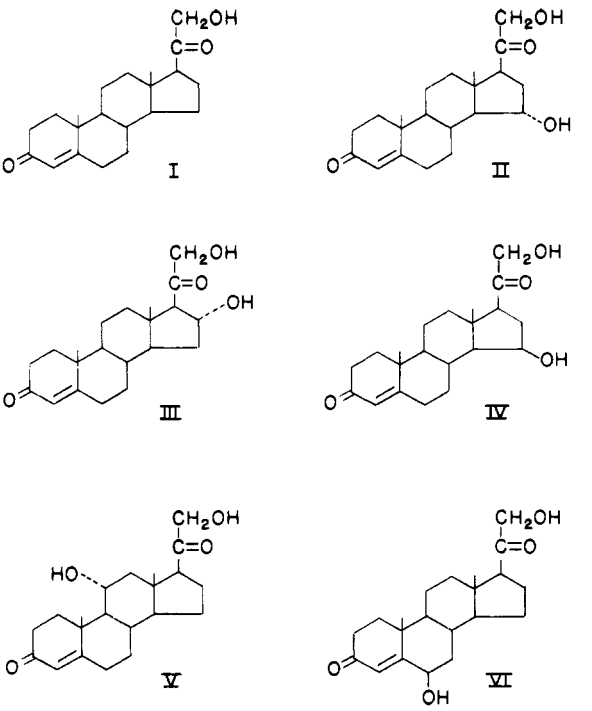

FIGURE 1: Structural formulas for substrate (I) and metabolites (II–VI).

The five metabolites, together with four other steroids of the same class, were characterized further by comparing their mobilities with that of compound I in four representative paper chromatographic systems. Since all of these substances differ from compound I only in possessing one additional hydroxyl group, it was advantageous to consider the results (Table II) in terms of ΔR_{M_9} values. This index, which was adapted by Bush (1961) from the original ΔR_M concept of Bate-Smith and Westall (1950), serves to express the effect of a single group, in this case the additional hydroxyl group, on mobility. As noted by Bush (1961, Table 2.2, pp. 87, 88), its effect is determined by its proximity to other groups and by its configuration. The values in Table II further illustrate these generalizations: the low values for steroids 5 and 9 are owing in the former case to the approach of the C-11 axial hydroxyl group to both methyl groups, and in the latter case to the tertiary nature of the hydroxyl group and to its approach to the C-20 carbonyl group. When the three epimeric pairs are considered, it is evident that, with the exception of the reversed values for compounds 2 and 3 in system 7, all conform to the rule of Savard (1953) which states that the equatorially oriented member of an epimeric pair is the less mobile (in this case possesses the larger ΔR_{M_9} value). But our results do not bear out the contention of Bush (1961) that the ΔR_{M_9} values for hydroxyl groups in "atypical" systems, namely, those based on *t*-butyl alcohol (such as systems 3 and 5), are from one-fourth to one-half those found in "typical" systems (represented by systems 7 and 8). It will be seen

from Table II that only two ΔR_{Mg} values, obtained by chromatographing steroids 4 and 8 in system 3, differ significantly from the corresponding values obtained with the other three systems. In reference to the present application, the data in Table II serve to confirm the configuration of C-15 assigned to the epimeric metabolites II and IV, and provide added proof that all of the isolated metabolites differ from compound I only in possessing a single additional hydroxyl group.

Discussion

Several factors combined to make paper chromatography ideally suited as a fractionating technique in these experiments. In contrast to the characteristics of surviving rat liver slices (Schneider and Horstmann, 1951), bullfrog liver slices have very little capacity to reduce double bonds or carbonyl groups. This reduced the fractionation problem to little more than the separation of the metabolites from one another and from extraneous material, and greatly simplified their detection on the chromatograms. Finally, suitable standards of reference were available as well as some information on their paper chromatographic characteristics.

The introduction of hydroxyl groups into the 11β- and the 17α-positions are essential steps in adrenal steroid biogenesis, but it has been difficult to determine the physiological ends served by those hydroxylations which occur in the liver. The meager evidence available from published studies, *in vitro* (in mammals, and irrespective of substrate or species selected), can be summarized by indicating that relatively few nuclear sites are involved, that these include the 2α, 2β, 6β, 7α, and 16α positions, and that the occurrence of a particular hydroxylation is influenced considerably by the substrate employed and greatly by the species selected.

The data in the present paper demonstrate that the liver of the bullfrog can hydroxylate compound I at five different nuclear positions[3] and that these hydroxylations occur singly rather than in multiple. Positions 11α, 15α, and 15β of compound I are not hydroxylated by enzyme systems derived from mammalian liver. Steroid hydroxylating systems in bullfrog liver thus differ from those in mammalian liver in their capacity to introduce hydroxyl groups into a greater number as well as certain "new" sites. We regard the apparently randomized hydroxylations characteristic of the bullfrog as a mark of the primitive, in the biochemical sense.

Some support for this view may be derived from the studies of Haslewood (1959), who has attempted to correlate the structures of the bile acids isolated from a variety of species with their taxonomic positions. The most primitive vertebrates secrete alcohols closely related to cholesterol, which may be hydroxylated in the side chain as well as in the nuclear positions. As the scale is ascended, the side chain is shortened, the terminal carboxyl group appears, and hydroxylation becomes limited to nuclear sites. The bullfrog secretes 3α,7α,12α-trihydroxycoprostanic acid and a little cholic acid, but principally 5α- and 5β-ranol, which are the C-5 epimers of 3α,7α,12α,24,26-pentahydroxycholestane.

The bullfrog adrenal can elaborate aldosterone and corticosterone (Ulick and Solomon, 1960; Carstensen *et al.*, 1959) but there is no proof that deoxycorticosterone also is synthesized at this site. Since the observed hepatic hydroxylations utilize a common substrate and presumably are effected by enzymatic approach both from the front or top side of the molecule (giving compounds IV and VI) as well as from its back or bottom side (forming compounds II, III, and V), two or more site-activating enzymes must be present in the tissue or are elaborated inductively in the course of the incubation.

Similar, smaller-scale incubations were carried out in which deoxycorticosterone was incubated with surviving liver slices from the red racer (*Masticophis flagellum piceus*) and the bullsnake (*Pituophis catenifer affinis*). In both cases metabolites II, III, V, and VI (the major product) were recovered and identified. Thus the hydroxylating characteristics of the bullfrog, a typical amphibian, are largely shared by two members of the class *Reptilia*. It has been shown that, like the bullfrog, the adrenals of two representative reptiles (a lizard, *Lacerta viridis*, and a snake, *Natrix natrix*) elaborate aldosterone and corticosterone (Phillips *et al.*, 1962).

Acknowledgment

We are most grateful to Dr. Robert Neher for the gift of the reference steroids, and to Beatrice S. Gallagher for determination of the infrared spectra of both the reference and isolated steroids. We are also grateful to the referees of this paper for their constructive suggestions.

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[3] For purposes of convenience in the present paper, the terms "position" or "site" are used to designate both the particular carbon substituted by the hydroxyl group and its configuration.

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Synthesis of Antigenic Branch-Chain Copolymers of Angiotensin and Poly-L-lysine*

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ABSTRACT: Two branch-chain copolymers comprising backbones of poly-L-lysine and branches of the physiologically active octapeptide angiotensin II were synthesized. In one of these, the amino-terminal end of angiotensin was coupled to poly-L-lysine via *m*-xylylene diisocyanate. In the other instance, carbodiimide condensation was performed between the carboxyl-terminal end of angiotensin and the ε-amino groups of poly-L-lysine.

Both polymers elicited in rabbit the production of antibody specific for angiotensin. Binding of isotopically labeled angiotensin to antibody was demonstrated by gel filtration. One of these branch-chain polymers is active as a pressor, while the other is not. The antibody does not appear to compete with angiotensin for its physiologic binding site.

Polypeptides of low molecular weight are usually poor antigens. Angiotensin, an octapeptide with molecular weight 1031, appears to be nonantigenic (Deodhar, 1960). In an effort to make antibodies directed against angiotensin, the molecule was coupled covalently to poly-L-lysine, itself nonantigenic, and the resultant complex was tested for antigenicity in rabbits. By appropriate techniques, angiotensin could be attached to the polyamino acid backbone via either the carboxyl- or amino-terminal end. Antibodies binding isotopically labeled angiotensin were demonstrated. The techniques involved in making antibodies against angiotensin have wide application in immunoassay and in studies directed toward the mechanism of action and biological properties of this and other low molecular weight polypeptide hormones.

Materials and Methods

Materials. Synthetic asparagine[1] valine[5] angiotensin II (Hypertensin Ciba, lot A-7930) was provided free of excipients.[1] This material was demonstrated to be homogeneous by amino acid analysis, by high-voltage electrophoresis in formic–acetic acid buffer, *p*H 1.9, at 98 v/cm, and by gel filtration on Sephadex G-25. Infrared spectroscopy showed no evidence of residual blocking groups. Poly-L-lysine·HCl was obtained from the Mann Chemical Co. (lot J-2094). The molecular weight of this material was 17,000 as determined by a short-column equilibrium ultracentrifugation (Richards and Schachman, 1959) using a partial specific volume of 0.79 (Friedman *et al.*, 1961). *m*-Xylylene diisocyanate was obtained from the Mann Chemical

* From the Cardiac and Hypertension Units, Medical Services, Massachusetts General Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass. *Received November 25, 1964.* Supported in part by grants (AI-0496, HE-06664, and HE-08896) from the National Institutes of Health, U.S. Public Health Service.

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[1] The authors are grateful to CIBA for generously supplying this material.