IDENTIFICATION OF 19-HYDROXYDEOXYCORTICOSTERONE IN REGENERATING RAT ADRENAL INCUBATIONS

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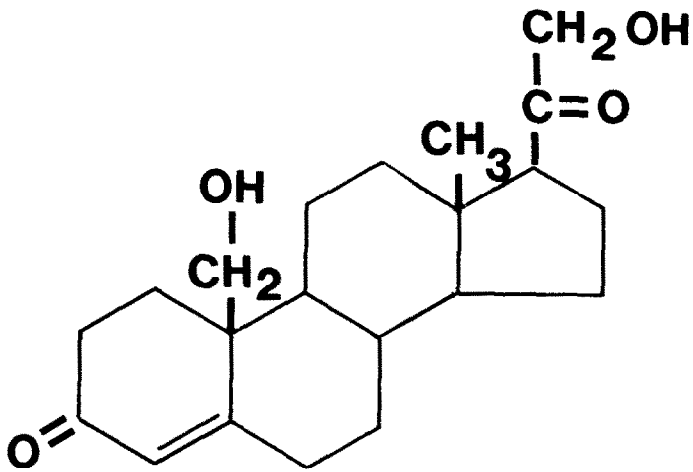
ABSTRACT

19-Hydroxydeoxycorticosterone (19-OH-DOC) was isolated from the incubation medium of enucleated rat adrenal glands during the early sodium retaining phase. Identification included comparison of chromatographic mobilites of parent and derivatized compound with standard prepared by the 21-hydroxylation of 19-hydroxyprogesterone and mass spectrometry. The possible role of 19-OH-DOC as a precursor is discussed.

INTRODUCTION

Following enucleation of the rat adrenal and uninephrectomy, there occurs regeneration of the gland and after 3 weeks severe hypertension ensues (1). Within 24 hours and for at least 2 weeks, adrenal venous steroid levels are markedly reduced (2). Beginning within 3-72 hours after enucleation and persisting to a lesser degree for 2 weeks, there is an impaired ability of the adrenal enucleated animal to excrete a light sodium load (3,4). Gaunt *et al* (5) provided indirect evidence that the sodium retaining phenomenon was produced by a humoral substance arising from the enucleated gland since ligation of the adrenal pedicle abolished the antinatriuresis, as did administration of spironolactone, suggesting that the salt retaining factor was a mineralocorticoid. That the salt retaining factor in the early phase of adrenal regeneration hypertension is not aldosterone was demonstrated by inhibiting the biosynthesis of aldosterone with the polysulfated polysaccaride, RO-1-807, metyrapone and aminoglutethimide which did not abolish the salt retention in response to bilateral enucleation (6,7). Subsequent studies from this laboratory (8) demonstrated that the enucleate

adrenal is capable of producing aldosterone when sodium is excluded from the diet. After 72 hours of high salt consumption following enucleation, aldosterone secretion is virtually nil. The present study describes an attempt to isolate and characterize the sodium retaining factor from the enucleated adrenal after the animals have been placed on a high salt diet for 72 hours after enucleation. This attempt has led to the isolation and identification of 19-OH-DOC (Figure 1), a unique secretory product of the enucleate adrenal.



19-OH-DOC

Figure 1. Chemical structure of 19-hydroxydeoxycorticosterone

MATERIALS AND METHODS

Pregnenolone-7-^{3}H (S.A. 25 Ci/mmole), obtained from New England Nuclear Corporation, was purified prior to use on a silica gel GF 375 micron plate in the thin layer system chloroform:acetone (95:5). The labeled material was located by scanning on a Packard Model 7201 radiochromatogram scanner, eluted and stored in ethanol. Unlabeled steroids used as reference standards were obtained from Steraloids. 19-Hydroxyprogesterone was generously supplied by Syntex Laboratories.

All solvents were distilled over anhydrous potassium carbonate with the exception of HPLC grade methanol and water. High pressure liquid chromatography (HPLC) involved a Dupont model 850 liquid chromatography employing a Zorbax C-18 column (Dupont).

Mass spectroscopy of substances obtained from the polar region from thin layer chromatograms from incubations of enucleated rat adrenal glands was performed using a Dupont DP-102 Analytical Mass Spectrometer. Mass spectroscopy of prepared standard 19-hydroxydeoxycorticosterone was performed using a Finnegan 3200 Mass Spectrometer through the courtesy of Dr. Kenneth I.H. Williams at the Worcester Foundation for Experimental Biology.

Animals: Male Sprague-Dawley rats, 200-250 gm body weight, were used for all studies. Adrenal enucleations were performed as described previously (6). All rats, enucleated and intact normals, were given 1% sodium chloride to drink and regular rat chow for three days prior to sacrifice and adrenal incubations.

Incubations of Enucleated and Normal Adrenals: The rats were sacrificed by suffocation in ether, the adrenals or regenerating capsules removed, trimmed of excess fat, quartered and placed in ice-cold Krebs Ringer bicarbonate buffer, pH 7.4, containing 200 mg% glucose. Ten rats' adrenals were incubated in 5 ml of buffer containing 1 mg dibutyryl cyclic AMP and 5 μCi ^{3}H-pregnenolone for three hours at 37°C in a forced air atmosphere. A total of 500 rats' adrenals for the adrenal enucleate study and ten normal rats' adrenals were incubated. The medium from 10 adrenal enucleated rats was kept separate and processed with the normal glands incubation medium to compare conversion products from labeled pregnenolone.

Product Isolation: The media were extracted twice with five volumes of methylene chloride which was evaporated to dryness in vacuo. The residue was partitioned between equal volumes of 80% aqueous ethanol and petroleum ether and the aqueous ethanol evaporated to dryness in vacuo. The residue was chromatographed on a 375 micron silica GF plate using as a solvent chloroform:ethanol (97:3) until the solvent front had reached 15 cm. Deoxycorticosterone (DOC) served as a standard marker. In the extracts kept separate (normal and enucleated adrenals) for tritium conversion studies, a 5 cm-wide scanning plate was used. The area from the origin to one cm below the DOC standard was eluted by suspending the silica gel in 5 ml water and extracting with 50 ml methylene chloride which was then washed with 5 ml water. The residue was dissolved in methanol and filtered through washed glass wool. An aliquot was subjected to gas chromatography on a 2 meter 3% OV101 column at 120°C and mass spectral analysis by electron impact of the emerging peaks. Another aliquot was chromatographed on a C-18 Zorbax ODS (octadecylsilane) column, using 60% aqueous methanol as the mobile phase with a flow rate of 1 ml per minute on a Dupont 850 HPLC. The polar area of the thin layer chromatogram from the incubation of normal glands and enucleated glands with tritiated pregnenolone was collected from the HPLC in 0.5 ml aliquots and the tritium analyzed by counting in a Packard tricarb scintillation counter after addition of instagel counting solution.

Preparation of 19-Hydroxydeoxycorticosterone: The adrenal glands from ten rats were homogenized in 6 ml Krebs-Ringer bicarbonate buffer (pH 7.4) and immediately centrifuged at 5,000 g for 15 minutes at 4°C. The nuclear sediment was washed with 4 ml of fresh buffer, recentrifuged, and the supernate pooled with the original. The combined supernate was centrifuged at 10,000 g to sediment the mitochondria. The supernatant microsomal fraction, containing 21-hydroxylase, was added to a flask containing 0.5 mg of 19-hydroxyprogesterone, glucose-6-phosphate (25 mg), NADP (5 mg), and glucose-6-phosphate dehydrogenase (25 units, Sigma Chemical Company). The mixture was incubated for four hours at 37°C in an air atmosphere. The incubation medium was extracted with methylene chloride which was washed once with water and then evaporated to dryness in vacuo. The residue was chromatographed on silica GF in the thin layer system benzene:acetone (1:1) with corticosterone (B) as a reference standard. Two major UV absorbing (UV+) bands were observed migrating with R_B=1.18 (unchanged 19-OH-P) and R_B= 0.89. The more polar substance was eluted and chromatographed on Whatman #1 paper (9) impregnated with 30% formamide in methanol using as a mobile phase butyl acetate:ethyl acetate:formamide:water (85:15:5:5). The UV+ and blue tetrazolium positive (BTZ+) area migrating with R_B=0.46 was eluted and chromatographed on a thin-layer celite plate impregnated with 6% propylene-glycol in acetone. The mobile phase was toluene standard with propylene glycol and corticosterone was used as a reference standard. The area with R_B=0.39 was eluted and acetylated in a mixture of acetic anhydride:pyridine (2:1) overnight. A UV+, BTZ+ zone was observed migrating with R_{DOC}=1.3 when chromatographed on silica gel in the thin layer system chloroform:ethanol (97:3). The underivatized compound was subjected to mass spectrometry by direct probe, using a Finnegan 3200 Mass Spectrometer with electron energy of 70 eV, ambient probe temperature, and ion source temperature at 90°C.

RESULTS

The results of the conversion of tritiated pregnenolone to products in enucleated and normal rat adrenals present in the polar area of the thin layer chromatogram after fractionation of HPLC are shown in Figure 2. Considerably less conversion to corticosterone and 18-OH-DOC (not separated in this system) is present in the enucleated adrenal incubation than in that from normal glands. As evidenced by the conversion pattern in the early part of the chromatogram (< 14 minutes), more polar products are formed in the enucleated adrenal than in the normal adrenal incubation. The material with the retention time of 12 minutes (not demonstrable in the normal gland incubation but present in the enucleate adrenal incubation) was identified as 11-dehydrocortico-

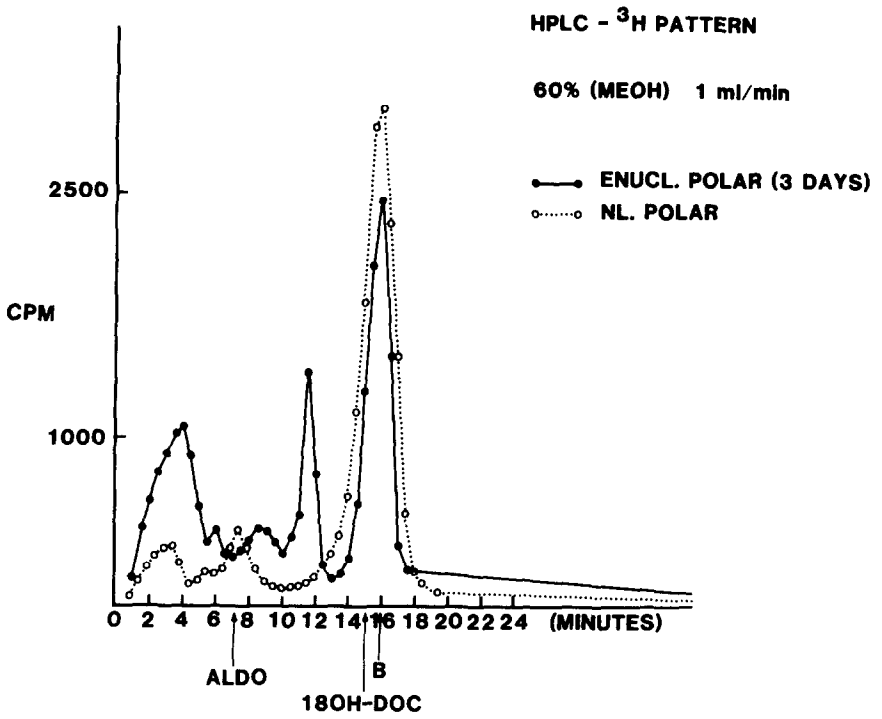


Figure 2. High pressure liquid chromatograph of polar eluate from thin layer chromatogram of normal rat adrenals and three day adrenal enucleated rat incubations

sterone by nuclear magnetic resonance and comparison with the authentic standard. The radioactive peak with the retention time of 9.2 minutes was collected and chromatographed in the systems indicated in the Methods Section for preparation of 19-OH-DOC. Identical mobilites were obtained for the radioactive rat substance and standard 19-OH-DOC as both the free compound and the acetate.

Table I compared the gas chromatography/mass spectra of a substance with a molecular ion at 346 obtained from the polar region of the thin layer chromatogram and standard 19-OH-DOC prepared from 19-OH-progesterone. Both spectra give a relatively strong molecular ion at 346 and base peak at 258 arbitrarily chosen as largest ion peak above

150. This ion at 258 may represent loss of the CH_2OH carbon 19 plus subsequent loss of C_3H_4O from carbons 1, 2 and 3 with an additional H ion. Comparison of this spectra's base peak with those of corticosterone and 18-OH-DOC (molecular weight = 346) published by Genard et al (10) shows little similarity. Although the ions at 315 exists in all spectra, it represents the major ion fragment of corticosterone with the next major ion at 269 which is not present in the spectrum of 19-OH-DOC. The base peak of 18-OH-DOC in the spectrum obtained by Genard et al (10) is at 299, which is not seen in 19-OH-DOC spectrum.

TABLE I

MASS FRAGMENT OF IONS OF STANDARD 19-OH-DOC AND RAT ENUCLEATED ADRENAL PRODUCT

Ion Molecular Weight	% of Base Peak 258 Rat	Standard
346 M+	53	25
315	53	90
286	59	25
270	35	30
259	47	35
258 (Base)	100	100
242	35	20
240	35	20

DISCUSSION

Chromatographic and mass spectral data of a compound isolated from the incubation medium of rat adrenals 72 hours after enucleation provides strong supporting evidence for the identification as 19-OH-DOC when compared with 19-OH-DOC prepared by microsomal 21-hydroxylation of 19-hydroxy-progesterone. The possible synthesis of 19-OH-DOC by normal rat adrenals as judged by the lack of conversion of tritiated pregnenolone to this compound during incubation.

Mattox (11) and Neber and Wettstein (12) have reported the isolation of 19-OH-DOC from extracts of beef and hog adrenal glands. The

amount of 19-OH-DOC that these investigators obtained was considerably less than the amount of aldosterone isolated from the extracts. Hayano and Dorfman (13) demonstrated the conversion of DOC to 19-OH-DOC by adrenal homogenates and Levy and Kushinsky (14) showed the conversion of progesterone to 19-OH-DOC by perfusing bovine adrenal glands with progesterone in blood.

The enucleated rat adrenal seems to be capable of forming considerably more 11-dehydrocorticosterone than the normal rat adrenal as evidenced by the conversion of tritiated pregnenolone used as a precursor (Figure 2). Although 11-dehydrocorticosterone is a known product of the rat adrenal gland, it is interesting that the enucleated rat adrenal gland exhibits increased 11β-hydroxysteroid dehydrogenase activity. The reason for this increased activity is unknown; however, the activity of other hydroxy-steroid dehydrogenases may also be increased. Although 19-oxo-DOC was not isolated and identified in these experiments, it is reasonable to assume that this steroid is present since the action of 19-OH-steroid dehydrogenase would yield this steroid. Since 19-oxo-DOC could serve as a precursor for the hypertensinogenic steroid 19-nor-DOC following decarboxylation, interest in this steroid is keen. 19-Nor-deoxycorticosterone has been demonstrated to exist in the urine of rats with adrenal regeneration hypertension by Gomez-Sanches et al (15). 19-Nor-DOC has sodium retaining ability greater than that of DOC, whereas 19-OH-DOC has only about 4% of the activity of DOC in a crude assay for sodium retention (11). Whether 19-nor-DOC is responsible for the sodium retaining activity which is exhibited in the early phase post-adrenal enucleation is unknown because this steroid has not been demonstrated in the

adrenal venous effluent of the enucleated adrenal or in the peripheral blood. We have been unable to show conversion of labeled pregnenolone to 19-nor-DOC in the incubations of adrenals enucleated 72 hours before sacrifice. The humoral factor(s) response which induces the early phase sodium retention is more polar than deoxycorticosterone or 19-nor-DOC as evidenced by the bioassay of eluates of regions from initial thin layer chromatographic separations (16). Whether these more polar steroids are converted in vivo in the bioassay procedure to 19-nor-DOC is not known. Furthermore, it is not certain that 19-nor-DOC is the only salt retaining metabolite formed in adrenal enucleation. 19-Nor-progesterone which can serve as a precursor to 19-nor-DOC (17) is a potent hypertensinogenic steroid when implanted subcutaneously in Alzet osmotic minipumps in rats (18). 19-Nor-progesterone possesses 55% of the hypertensinogenic activity of aldosterone (18).

STEROID NOMENCLATURE

Aldosterone = 11β,21-dihydroxy-18-oxo-4-pregnene-3,20-dione

19-Hydroxydeoxycorticosterone (19-OH-DOC) = 19,21-dihydroxy-4-pregnene-3,20-dione

19-Hydroxyprogesterone = 19-hydroxy-4-pregnene-3,20-dione

18-Hydroxydeoxycorticosterone (18-OH-DOC) = 18,21-dihydroxy-4-pregnene-3,20-dione

Corticosterone (B) = 11β,21-dihydroxy-4-pregnene-3,20-dione

19-Oxodeoxycorticosterone (19-oxo-DOC) = 21-hydroxy-19-oxo-4-pregnene-3,20-dione

11-Dehydrocorticosterone (A) = 21-hydroxy-4-pregnene-3,11,20-trione

Pregnenolone = 3β-hydroxy-5-pregnen - 20-one

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