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METABOLISM OF 11-DEOXYCORTICOSTERONE BY HAMSTER ADRENAL MITOCHONDRIA

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Summary—Metabolism of 11-deoxycorticosterone (DOC) by hamster adrenal mitochondria gives 19-hydroxy-DOC and corticosterone (via 11-hydroxylation) in approximately equal yields. The ratio of 19- to 11-hydroxylation was invariant with changes in concentration of substrate or a competitive inhibitor. It is most likely, therefore, that a single 11,19-hydroxylase catalyzes both oxidations. Both primary products are further oxidized to the corresponding carbonyl analogs, 19-oxo-DOC and 11-dehydrocorticosterone, at rates that are approx. 20% of their rates of formation. The oxidation of 11-dehydrocorticosterone is catalyzed by a dehydrogenase utilizing either NAD or NADP while the oxidation of 19-hydroxy-DOC is catalyzed by an oxidase requiring NADPH. The 11-dehydrocorticosterone is stable in this enzyme preparation while 19-oxo-DOC is metabolized to two additional products, which are tentatively identified as 19-oic-DOC and 19-norcorticosterone. 19-nor-DOC was found to be hydroxylated at a rate that is 20% faster than the rate for DOC under the same conditions. It is therefore possible that 19-norcorticosterone can arise from 19-oic-DOC via decarboxylation to 19-nor-DOC and subsequent 11-hydroxylation, but the kinetics of its formation suggest that it may actually be formed directly from 19-oxo-DOC without free intermediates. 4-Androstene-3,17-dione and 17-hydroxy-DOC were also substrates for this 11,19-hydroxylase, but 18-hydroxy-DOC was not. Maintenance of hamsters on a low sodium diet had no effect on the metabolism of DOC by the isolated adrenal mitochondria.

INTRODUCTION

Oxidative removal of the 19-methyl of the steroid nucleus can occur via a nonaromatizing pathway that proceeds by successive oxidations to the 19-alcohol, 19-aldehyde, and 19-acid. The 19-acid is subsequently decarboxylated to give the 19-nor steroid. 11-Deoxycorticosterone (DOC) is metabolized in this manner to 19-nordeoxycorticosterone (19-nor-DOC), and this metabolite has been implicated in certain types of hypertension [1].

Given the possible physiological significance of the pathway for 19-demethylation, we have been interested in the enzymology of the pathway and in particular in the first enzyme of the pathway, which is a 19-hydroxylase. This enzyme is a minor activity in most species but is abundant in the adrenals of hamsters [2]. We have, therefore, studied the 19-hydroxylation of DOC by mitochondria from hamster adrenals, comparing the 19-hydroxylase to the equally abundant 11-hydroxylase, and have traced the metabolic fate of the initial hydroxylation products from these two reactions in the mitochondrial preparation.

EXPERIMENTAL

[^{3}H]DOC was from Amersham Corp. (Arlington Heights, IL) and other radiolabeled steroids were from NEN Research Products (Boston, MA). Samples of 19-hydroxy-DOC were generously provided by Drs James Melby (Boston University Medical Center, Boston, MA) and Cecil Robinson (Johns Hopkins University, Baltimore, MD). Samples of 19-oxo-DOC and 19-nor-DOC were provided by Dr Cecil Robinson. Other unlabeled steroids and biological reagents were from Sigma Chemical Co. (St Louis, MO).

Syrian golden hamsters (Sprague–Dawley Harland, Indianapolis, IN) were maintained routinely on a normal rodent diet or, when indicated, for 8 weeks on a Hartcroft–Eisenstein sodium deficient rat diet (ICN Nutritional

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Biochemicals, Cleveland, OH). Whole adrenals were removed and homogenized with a Teflon pestle in a conical centrifuge tube in 0.25 M sucrose, 0.1 M KCl, 10 mM potassium phosphate, 1 mM EDTA, 10 mM DTT, pH 8.0. Mitochondria were prepared by differential centrifugation, discarding the 800 *g* pellet and collecting the 30,000 *g* pellet, which was washed once and resuspended in storage buffer (0.25 M sucrose, 20 mM Tris, 5 mM EDTA, pH 7.4). The mitochondria were stored at a protein concentration of 2 mg/ml at −78°C. The steroid-metabolizing enzyme activities were stable under these conditions for at least 3 months and could be repeatedly thawed and refrozen.

Enzyme assays were performed at 25°C in 0.2 ml of assay buffer (0.1 M Tris, 1 mM EDTA, pH 8.0, and routinely containing 1 mM NADPH unless otherwise indicated). Labeled and unlabeled steroids in organic solvents were combined, evaporated under argon, then redissolved in assay buffer and used with labeled steroids at 0.2 mCi/assay and unlabeled steroids at the concentrations indicated. Assays were initiated by addition of enzyme (*ca.* 10 μg of protein) and quenched by addition of an equal volume of 20% CH_3CN–2% acetic acid. The quenched assay mixture was centrifuged for 2 min at 15,000 *g* and analyzed routinely by liquid chromatography on two Bondapak C_{18} Radial Pak columns in series (10 μm particles, 0.8 × 10 cm each, Millipore Corp., Milford, MA). Chromatographic buffer A was 10% CH_3CN–0.1% acetic acid and buffer B was 80% CH_3CN–0.1% acetic acid. The columns were eluted at a flow rate of 1 ml/min with a linear gradient from 37 to 46.4% buffer B over 36 min followed by 100% B. These conditions were optimized for separation of observed products.

A second chromatographic elution protocol was used to confirm peak assignments under conditions optimized to resolve known steroid standards. Assay samples were extracted with ethyl acetate following addition of radiolabeled standards, dried under N_2 at 30–40°C, and redissolved in 1:1 CH_3CN–10 mM sodium acetate, pH 6.0. The same columns as above were used; buffer A contained 10% CH_3CN and buffer B contained 80% CH_3CN in 10 mM sodium acetate, pH 6.0. Linear gradients were used for the elution profile: 35 to 45% B at 0 to 8 min, 45 to 60% B from 8 to 13 min, 60% B continued to 20 min, 60 to 70% B from 20 to 28 min, 70 to 80% B from 28 to 29 min, 80% B continued to 36 min. The flow rate was also varied: 1.5 ml/min from 0 to 7 min, 1 ml/min from 8 to 13 min, 0.6 ml/min from 14 to 20 min, and 1 ml/min from 21 to 36 min. Each change in flow rate was accomplished by a linear gradient over 1 min. The column was reequilibrated for 8 min at 1.5 ml/min between runs. The following steroids were resolved (elution times in min): 18-hydroxycorticosterone (9.5), aldosterone (12.0), hydrocortisone (16.0), 19-hydroxy-DOC (18.6), cortisone (19.2), 18-hydroxy-DOC (23.8), 11-oxo-DOC (28.4), corticosterone (29.9), 19-oxo-DOC (31.8), 19-nor-DOC (35.9), and DOC (38.4).

Unlabeled steroids were detected by their absorbance at 240 nm. The extinction coefficients for derivatives of DOC were assumed to be similar to that of DOC ($\epsilon = 17{,}2000\ M^{-1}\ cm^{-1}$). Radioactivity was detected with a Flo-One scintillation detector equipped with a 1 ml flow cell (Radiomatic Instruments and Chemical Co., Meridan, CT). Recovery of total radioactivity was used as the internal standard for spectrophotometric quantitation. Liquid chromatography–mass spectrometry (LC–MS) was performed with a Hewlett Packard 5970 mass-selective detector mounted in a Vestec 101 TSP interface with discharge mode ionization.

RESULTS

A chromatogram showing products obtained from incubation of [^{3}H]DOC with hamster adrenal mitochondria is presented in Fig. 1. The reaction had a broad pH optimum from pH 7.5

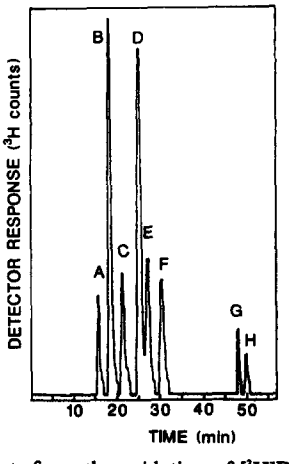

Fig. 1. Products from the oxidation of [^{3}H]DOC catalyzed by hamster adrenal mitochondria. Analysis was by the shorter HPLC protocol described in the Experimental section. Peak identities are as follows: (A) 19-norcorticosterone (tentative), (B) 19-hydroxy-DOC, (C) 19-oic-DOC (tentative), (D) 11-dehydrocorticosterone, (E) corticosterone, (F) 19-oxo-DOC, (G) 19-nor-DOC, and (H) DOC.

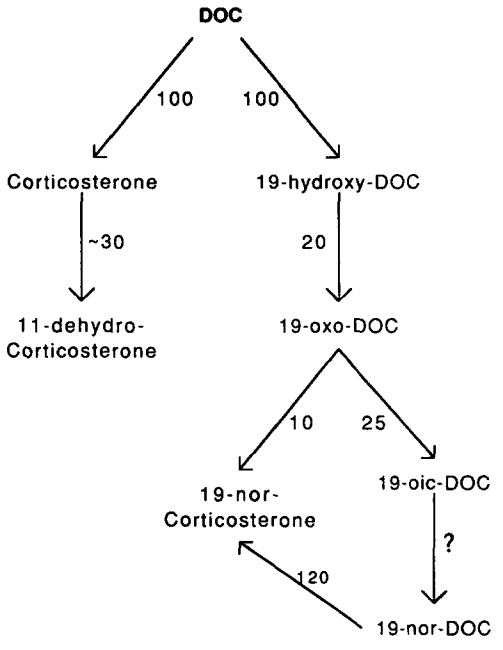

Fig. 2. Pathway and relative rate constants for the metabolism of DOC. Products were identified as described in the text. The assignment of 19-oic-DOC and 19-norcorticosterone is tentative. The rate of oxidation of corticosterone was variable (see text).

to 9.0 with respect to disappearance of DOC, and pH 8 was chosen for characterization of the reaction. The products arose exclusively from successive oxidations at the 11- and 19-positions of DOC. The metabolic pathway and relative rates for each step in the pathway are summarized in Fig. 2. Products were identified as follows and characterized in each case by coelution with standards by 2 HPLC protocols and by mass spectral analysis, unless otherwise specified.

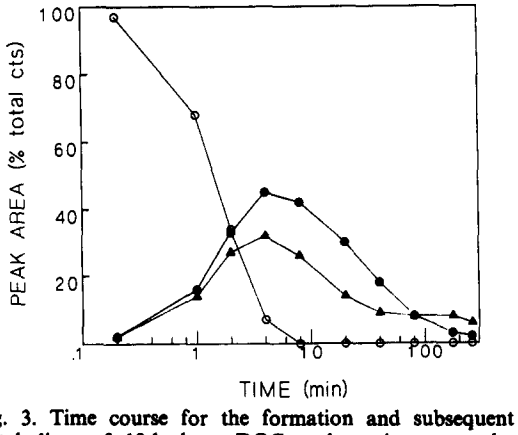

Fig. 3. Time course for the formation and subsequent metabolism of 19-hydroxy-DOC and corticosterone by adrenal mitochondria. [^{3}H]DOC, 20 nM, was incubated with adrenal mitochondria from hamsters on a normal diet at a protein concentration of 30 μg/ml. (○) DOC, (●) 19-hydroxy-DOC, (▲) corticosterone.

The relative amounts of the various products changed during the course of the reaction, and peaks B and E were the only peaks detected early in the reaction. Peak B is 19-hydroxy-DOC, and peak E is corticosterone. A representative time course for formation and disappearance of these primary metabolites is shown in Fig. 3. The rate of formation of 19-hydroxy-DOC was 5.5 times faster than its rate of degradation. The rate of formation of corticosterone was only 3 times its rate of disappearance in the experiment shown, but the rate of disappearance was variable (see below). As further confirmation of the identities of the 19-hydroxy-DOC and corticosterone peaks, unlabeled 19-hydroxy-DOC or corticosterone were incubated concurrently with [^{3}H]-DOC. The rates of disappearance of the unlabeled steroids were the same as the rates for the corresponding labeled metabolites formed *in situ* from [^{3}H]DOC (data not shown), and the unlabeled materials gave the expected final product profiles (see below).

The major metabolite derived from corticosterone in this system was 11-dehydrocorticosterone (peak D, Fig. 1), which was stable under the assay conditions.

The product initially formed from 19-hydroxy-DOC (peak F, Fig. 1) was 19-oxo-DOC. Unlike 11-dehydrocorticosterone, 19-oxo-DOC was further metabolized. The rate of disappearance was approximately twice the rate of formation so that only small amounts of this intermediate were observed at steady-state. 19-Oxo-DOC was converted to two major products, peaks A and C. In order to confirm that peaks A and C are formed via the 19- and not the 11-hydroxylation pathway, [^{14}C]DOC and [^{3}H]corticosterone were incubated together under normal assay conditions, chromatographic fractions were collected (0.5 min/fraction), and the ^{14}C/^{3}H ratio was determined for each peak. Peaks A and C contained only ^{14}C, as did the peaks attributed to 19-hydroxy-DOC and 19-oxo-DOC.

Peak C is tentatively identified as 19-oic-DOC. An MH^+ ion of m/z 361 was observed for this steroid by LC–MS, but a standard was not available. Likewise, the identity of peak A has not been firmly established by comparison with a known standard, but analysis by LC–MS showed that the molecular weight of this product differs from that of 19-oxo-DOC by the loss of 12 mass units. This would be consistent with the loss of the CHO group at position 19 and subsequent hydroxylation of the product,

reasonably at position 11 to give 19-nor-corticosterone.

The identification of 19-nor-corticosterone as a possible product suggested that this product might be formed via hydroxylation of 19-nor-DOC. When unlabeled 19-nor-DOC and [^{3}H]DOC were incubated together with this enzyme preparation, it was demonstrated that 19-nor-DOC is metabolized to a single product and that the rate of metabolism was 25% faster for 19-nor-DOC than for DOC (Fig. 5). The mass spectrum of the product from 19-nor-DOC agreed with that for peak A and was consistent with a single hydroxylation (M + H and M + H-H_2O peaks were observed). Although these results would be consistent with the formation of peak A by the expected pathway via 19-oic-DOC and 19-nor-DOC, there was no precursor–product relationship in the kinetics of formation of 19-oic-DOC and peak A. Rather, the ratio of the two products had a constant value of 2.5 ± 0.3 over the course of formation and loss of 19-oxo-DOC. Both 19-oic-DOC and peak A appear kinetically, therefore, to be formed directly from 19-oxo-DOC. Further investigation will be required to firmly establish the pathway for formation of the hydroxylation product of 19-nor-DOC.

Peak G was identified as 19-nor-DOC. This peak was normally not observed, except in samples that sat the longest between quenching and analysis of reaction mixtures. It most probably arises only via slow decarboxylation of 19-oic-DOC after quenching the assays.

No products arising from hydroxylation at the 18-position were observed. This includes 18-hydroxy-DOC, which elutes between peaks C and D, and 18-hydroxycorticosterone and aldosterone, which elute before peak A. The limit of detection for these steroids was approx. 1% of total products. Formation of aldosterone via hydroxylation at C-18 can be increased at least 10-fold in rats if they are maintained on a sodium-deficient diet [3]. No induction of any products from 18-hydroxylation was observed in hamsters maintained on the same diet for 8 weeks, however. In addition, no significant change in either the product profile or the specific activity of 19- and 11-hydroxylation (relative to mitochondrial protein) was observed in hamsters on the low sodium diet. Since the absence of 18-hydroxylation in the adrenal is an unusual observation, adrenals were isolated a second time from hamsters on a low sodium diet, and immediately after preparation, the supernatant from a low speed centrifugation (800 *g*) was incubated with [^{3}H]DOC. In this assay, 94% turnover of DOC was achieved at 30 min, but no products of 18-hydroxylation were observed at 30, 60, or 120 min. Further, the fractions from the 30 min assay that coeluted with authentic aldosterone, 18-hydroxycorticosterone, and 18-hydroxy-DOC were collected and rechromatographed to increase sensitivity of detection of any radioactive products. Still no trace of these products was observed.

Studies of the product distribution from the oxidation of DOC suggested that the 11- and 19-hydroxylase activities might be expressed by a single enzyme. For example, if there was only one enzyme, then the ratio of 11- vs 19-hydroxylation would be determined by the partitioning of the enzyme–DOC complex to 19-hydroxy-DOC and corticosterone, and this partitioning would be independent of the substrate concentration. On the other hand, if the 11- and 19-hydroxylases were different enzymes, then the product ratio would vary with substrate concentration, unless the K_m of the substrate for both enzymes were fortuitously the same. Similarly, an inhibitor of the hydroxylation reactions would not affect the product ratio if the 11- and 19-hydroxylases were the same enzyme but would affect the ratio if they were different enzymes and the inhibitory constants for the two enzymes were different.

Data for formation of 19-hydroxy-DOC at a range of concentrations of DOC from 0.02 to 25 μM is presented in Fig. 4. The K_m for DOC

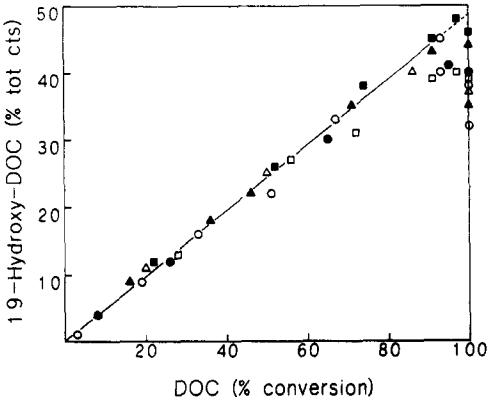

Fig. 4. Formation of 19-hydroxy-[^{3}H]DOC as a function of the concentration of DOC or added unlabeled 19-hydroxy-DOC. The 19-hydroxy-[^{3}H]DOC concentration is expressed as a fraction of the total product formed from [^{3}H]DOC. Concentration of DOC (μM): (○) 0.02, (●) 0.04, (△) 0.9, (▲) 5, (□) 25, (■) 0.2 plus 19 μM 19-hydroxy-DOC. The decrease in levels of 19-hydroxy-DOC at long reaction times (> 80% conversion of DOC) is due to its oxidation to 19-oxo-DOC.

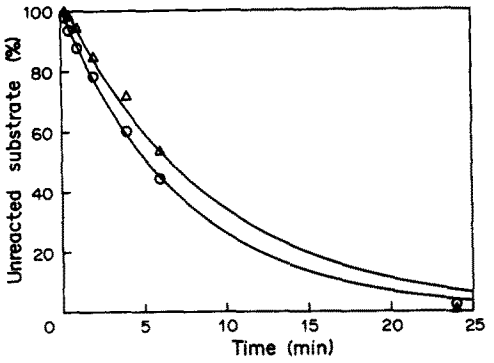

Fig. 5. Relative rates of oxidation of DOC and 19-nor-DOC. Oxidation of a mixture of [^{3}H]DOC (△) and 90 μM unlabeled 19-nor-DOC (○) was quantitated as loss of substrate vs time. Lines were calculated by least squares to fit a single exponential, and first-order rate constants were 0.13 and 0.11 h^{-1} for 19-nor-DOC and DOC, respectively.

is 0.8 μM under these conditions. Also included in this data is one experiment with the product 19-hydroxy-DOC, 19 μM, added as a competitive inhibitor, $K_i = 13$ μM. Data obtained under this wide variety of conditions was normalized by plotting the yield of 19-hydroxy-DOC as percent of total products versus the percentage of DOC oxidized. Further oxidation of 19-hydroxy-DOC and corticosterone is slow enough that it is insignificant until at least 75% conversion of the DOC has been turned over. In the region 0–75% conversion of DOC, therefore, 19-hydroxy-DOC and corticosterone account for essentially all of the products observed, and the percentage yield of 19-hydroxy-DOC is a direct measure of the partition ratio for product formation. The yield of 19-hydroxy-DOC is clearly independent of the concentration of DOC or inhibition by 19-hydroxy-DOC. It is very likely, therefore, that a single enzyme is responsible for both 11- and 19-hydroxylations.

Table 1. Cofactor requirements for oxidation of 19-hydroxy-DOC and corticosterone to 19-oxo-DOC and 11-dehydrocorticosterone, respectively

Cofactor	19-Hydroxy-DOC (% conversion)	Corticosterone (% conversion)
NADPH	72	4
NADP	4	64
NAD	5	79

[^{3}H]Corticosterone and 19-hydroxy-DOC, both 19 mM, were incubated together for 4 h with adrenal mitochondria at 0.3 mg/ml. The concentration of cofactors was 1 mM. An NADPH regenerating system (3 mM glucose-6-phosphate and 1 IU/ml glucose-6-phosphate dehydrogenase) was included in assays with NADPH as cofactor. Reported conversions represent product formed in excess of conversion in a control with no cofactor present. In the controls, 26% of the corticosterone was oxidized while no significant oxidation of 19-hydroxy-DOC occurred.

In contrast, the oxidation of 19-hydroxy-DOC and corticosterone to the oxo compounds is catalyzed by different enzymes. The data in Table 1 demonstrate that the oxidation of 19-hydroxy-DOC is supported by NADPH, as would be expected if the oxidase were a cytochrome *P*450. The oxidation of corticosterone is supported by NAD and NADP but not by NADPH. This reaction is presumably catalyzed by an NAD-dependent dehydrogenase. The rate of oxidation of corticosterone varied when several enzyme preparations were compared, so that the enzyme catalyzing this reaction may not be a mitochondrial enzyme but may be an enzyme from another subcellular pool contaminating the mitochondrial preparation.

Hamster adrenal mitochondria also catalyzed the oxidation of 4-androstene- 3,17-dione at a rate 50% of that for DOC. Only two major products were formed, and these coeluted with 11- and 19-hydroxyandrostenedione standards on reverse phase chromatography. The ratio of 19- to 11-hydroxylation products was 2.5:1. These products were relatively stable, although some further metabolism was observed at a rate $<10\%$ of the rate of formation. 17-Hydroxy-DOC was turned over at a rate comparable to that of DOC to give three major products that were not characterized. In contrast, when 18-hydroxy-[^{3}H]DOC was incubated together with [^{14}C]DOC, no significant metabolism of the 18-hydroxy-DOC was observed in the time required to achieve turnover of 95% of the DOC.

DISCUSSION

Our results show that hamster adrenal mitochondria catalyze the oxidation of DOC at carbons 11 and 19. The 18- and 19-positions of DOC are proximate to the 11-position in space, and it is reasonable to expect that a steroid 11-hydroxylase would show some reactivity at the 18- or 19-position [4]. The purified cytochrome $P450_{11\beta}$ from bovine adrenals, for example, has been shown to hydroxylate DOC at all three positions and to hydroxylate 4-androstene-3,17-dione at both the 11- and 19-positions [5–7]. Although the hydroxylase from hamster adrenals has not been purified, the kinetics of hydroxylation of DOC presented herein strongly suggest that a single hydroxylase catalyzes oxidation of DOC at both the 11- and 19-positions. The hamster and bovine enzymes are, therefore, qualitatively similar, although 19-hydroxylation is quantitatively a minor pathway

for the bovine enzyme and the 18-hydroxylation is not observed with the hamster enzyme. Drummond *et al.* [8] have recently reported that 11- and 19-hydroxylation of DOC is similarly accomplished by a single enzyme in gerbil adrenals, based on comparable inhibition of the two reactions by chemical mediators and by polyclonal antibodies.

Our results show that 19-hydroxy-DOC is oxidized to 19-oxo-DOC in the presence of hamster adrenal mitochondria and that this oxidation appears to be mediated by a cytochrome *P*450, since it requires NADPH. In comparison, purified cytochrome $P450_{11\beta}$ from both porcine and bovine adrenals will oxidize 18-hydroxycorticosterone to the 18-oxo-corticosterone product, aldosterone [9]. The bovine enzyme also catalyzes the oxidation of 19-hydroxy-DOC to 19-oxo-DOC [6, 7, 10, 11]. It is probable, therefore, that the hamster 11,19-hydroxylase itself catalyzes the oxidation of 19-hydroxy- to 19-oxo-DOC, and preliminary results (not shown) indicate that inhibitors of [^{3}H]DOC hydroxylation slow the oxidation of 19-hydroxy-[^{3}H]DOC equally.

The oxidation of corticosterone to 11-dehydrocorticosterone has been observed in diverse tissues in many species. The enzyme that catalyzes this reaction is a membrane-bound dehydrogenase that utilizes a nicotinamide cofactor and is commonly associated with microsomes [12–16]. The enzyme that catalyzes the oxidation of corticosterone in hamster adrenal preparations is similar in characteristics to this ubiquitous enzyme, including the fact that the activity requires NAD^+ or $NADP^+$. In addition, the results suggested that the dehydrogenase most probably arises from contamination of the mitochondria by another subcellular pool. It is reasonable that this would represent microsomal contamination, although this has not been confirmed with marker enzymes.

The function of the 19-hydroxylation of DOC is generally believed to be the formation of 19-nor-DOC, a potent mineralocorticoid [17]. 19-Nor-DOC is believed to be formed via successive oxidations of the 19-methyl to give 19-oic-DOC in the adrenal and subsequently 19-nor-DOC. The decarboxylation in the last step has been thought to occur outside the adrenal since 19-nor-DOC is normally not observed as a product in this tissue [18]. We have observed each of the required steps of this system in the hamster adrenal, but an additional steroid, tentatively identified as 19-norcorticosterone, was also observed. The latter product would be predicted by prior observations of the oxidation of 19-nor-DOC to 19-norcorticosterone [19]. The data suggest that this product is formed directly from 19-oxo-DOC in the hamster adrenal but also clearly show that 19-nor-DOC would be oxidized to this product more rapidly than it would be formed. It would appear, therefore, that the hamster adrenal 19-hydroxylase system may be designed to produce 19-norcorticosterone rather than 19-nor-DOC, and it is relevant that 19-norcorticosterone has also been shown to be a mineralocorticoid [20, 21].

The kinetics of the 19-hydroxylation pathway are unusual for a system whose function is to produce 19-nor-DOC or 19-norcorticosterone. The first committed step of the pathway is the fastest step, while the second step is significantly slower and is rate-limiting (Fig. 2). In addition, if our preliminary indications are valid, then the same enzyme catalyzes both the first and the second oxidations, and it is not obvious how the rate of the second could be increased relative to the first. In order to test whether it is possible to modulate the rate of the second step, we felt that the metabolic patterns might be different for hamsters on a low sodium diet. No changes were observed in hamsters that were maintained on a low sodium diet for 8 weeks, however. This is in sharp contrast to the rat, in which the synthesis of aldosterone and 18-hydroxycorticosterone from corticosterone is markedly increased in response to the same diet [3]. It is not clear, therefore, what changes in the 19-hydroxylation pathway can be expected in the hamster and what the stimuli for these changes might be. In addition, our inability to detect aldosterone synthesis raises the question whether this pathway exists in the hamster adrenal and whether aldosterone plays a role as a mineralocorticoid in the hamster. We have no data that would confirm *in vivo* the lack of aldosterone production that we observe *in vitro*, however, and this will be critical to the validation of our results.

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