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The 19-Hydroxylase of the Gerbil Adrenal Gland: A Mitochondrial Enzyme (40935)

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Abstract. The adrenal gland of the Mongolian gerbil produces nearly equal amounts of 19-hydroxy- and of 11β-hydroxycorticosteroids (Oliver and Peron, *Steroids* **4**, 351 (1964)). The intracellular location of the 19-hydroxylase (OHase) was studied by determining the activities of both the 19- and 11β-OHase enzymes in gerbil adrenal homogenate and subcellular fractions. Adrenal preparations were incubated *in vitro* in the presence of exogenous steroid precursor, androstenedione (A), testosterone (T), or deoxycorticosterone (DOC), plus a source of reducing equivalents. When adrenal homogenate was incubated with A plus isocitrate, the only two products detected were 19-hydroxy A and 11β-hydroxy A; the two were formed at virtually identical rates. With subcellular fractions, the capacity for 19-hydroxylation coincided with that for 11β-hydroxylation; the highest activities were in adrenal mitochondria. For adrenal mitochondria the two hydroxylation reactions were (1) enhanced by the addition of Ca^{2+} to incubations containing NADPH, (2) supported by either isocitrate or succinate as a source of reducing equivalents, and (3) present at nearly the same levels of activity. The 19- and 11β-OHase activity in other subcellular fractions appeared to be of mitochondrial origin. The cytosol was found to contain a C-17 oxidoreductase catalyzing the interconversion between A and T in the presence of the appropriate NADP(H) cofactor. It was concluded that the 19-OHase of the gerbil adrenal gland is primarily a mitochondrial enzyme. The localization and activities of both the 19- and 11β-OHases account for the relative amounts of the major corticosteroids found in the blood of the gerbil.

After Barber and Ehrenstein (1) reported the synthesis of 19-hydroxy-11-deoxycorticosterone (19-OH DOC), bovine adrenal glands were found to contain that compound and to produce 19-hydroxy (19-OH) steroids *in vitro* (2–5). Since that time, the formation of 19-OH steroids *in vitro* has been demonstrated in adrenal preparations of several other animal species including duck, rat, hamster, hog, and human fetus (5–9). The adrenal gland of the Mongolian gerbil, *Meriones unguiculatus*, is unusual in producing significant amounts of 19-OH corticosteroids both *in vivo* and *in vitro*. Oliver and Peron (10) first demonstrated that nearly equal amounts of cortisol and 19-hydroxy-11-deoxycortisol (19-OHDC) were present in peripheral and adrenal venous plasma of the gerbil. Though some studies have been carried out on corticosteroidogenesis by gerbil adrenal tissue incubated *in vitro* (10–13), the subcellular location of the 19-hydroxylase in the adrenal gland of this species has yet to be established. This investigation was undertaken to establish the localization of that enzyme. It was postulated that in the adrenal gland of the gerbil, as in the duck (1), the 19-hydroxylase would be a mitochondrial enzyme. The initial approach taken to test the hypothesis was an evaluation of the relative activities of the 11β- and 19-hydroxylase enzymes in gerbil adrenal preparations.

Materials and methods. Laboratory-reared Mongolian gerbils, segregated as to sex, were maintained in colony cages and given Purina laboratory feed and tap water *ad libitum*. The animal room was maintained at 23° on a 14-hr light–10-hr dark cycle. For each experiment, adrenal glands were collected from 10 to 20 adult male animals at 9 AM on the morning of the experiment. After the animals were decapitated, adrenal glands were quickly removed and placed in ice-cold 0.25 *M* sucrose. The glands were trimmed of adhering tissue, and the pooled glands were blotted dry and weighed to the nearest 0.1 mg. These glands were gently homogenized in 7 to 10 vol of

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0.25 *M* sucrose containing 30 m*M* Hepes buffer, pH 7.4, in a glass tissue grinder using four or five strokes of a motor-driven Teflon pestle. For incubations, the homogenate was diluted to contain the equivalent of 5 to 10 mg wet weight of adrenal tissue per 0.1 ml of homogenate. Subcellular fractions were obtained by differential centrifugation of the homogenate using the procedure described by Peron and McCarthy (14).

Incubations, *in vitro,* were carried out in 20-ml beakers at 37° in air with agitation using a Dubnoff incubator. The incubation media consisted of 0.154 *M* KCl, 30 m*M* Hepes buffer, pH 7.4, and 5 m*M* $MgCl_2$ with either 1 or 2 m*M* NADPH or 10 m*M* oxidizable substrate, isocitrate, malate, or succinate, as a source of reducing equivalents. In some cases, 10 m*M* Ca^{2+} was added to incubations containing NADPH. Androstenedione or testosterone was used as exogenous steroid precursor. A 9 m*M* steroid solution was prepared by mixing unlabeled steroid with ^{3}H-labeled steroid (New England Nuclear) in a solution of ethanol–propylene glycol (1:1, v/v). Addition of 0.01 or 0.02 ml of the stock solution to each milliliter of medium provided a final concentration of 90 to 180 nmole of precursor steroid containing 0.05 to 1 μCi radioactivity per milliliter of incubate. Incubation reactions were carried out for intervals of 5 to 30 min, taking appropriate zero-time samples; reactions were stopped by freezing.

Aliquots of the incubate were extracted with 5 vol of distilled dichloromethane. At the extraction step carrier and recovery steroid standards were added. Carrier standards were 1 to 5 μg of the appropriate 11β-OH and 19-OH derivatives of androstenedione or testosterone; the recovery standard was a trace amount of [^{14}C]-androstenedione. The dried extracts were subjected to thin-layer chromatography (TLC) on glass plates coated with silica gel HF_{254}. Two chromatography solvent systems proved equally effective in separating steroids extracted from the reaction mixture. Conventional TLC separation was carried out on 10 × 20-cm glass plates with multiple development in a mixture of cyclohexane–ethyl acetate (1:1, v/v). Separation of steroids by short bed/continuous development TLC (St. Regis Co.) was achieved using glass strip TLC plates (2.5 × 10-cm, Analtech) with development for 45 min in a system consisting of cyclohexane:dichloromethane:methanol:water (15:14:0.95:0.5, by volume). The separated steroids were visualized under ultraviolet light, fluorescent areas of the silica gel were scraped into glass vials, scintillation cocktail was added, and the samples were counted in a Beckman LS 3155T scintillation counter with automatic quench control. Recovery of standard ranged from 83 to 103%. The data were expressed as nanomoles of steroid converted per time interval per milligram wet weight adrenal tissue (or per milligram of protein). Protein determinations were carried out using the method of Lowry *et al.* (15).

Results. When gerbil adrenal homogenates were incubated with androstenedione (A) in the presence of isocitrate, the only two products detected were 19-hydroxyandrostenedione (19-OH A) and 11β-hydroxyandrostenedione (11β-OH A). The two products were formed at virtually identical rates in a reaction that was linear for 30 min (Fig. 1). In a similar system, incubation in the presence of NADPH + Ca^{2+}, the conversion of A to 19-OH A and 11β-OH A was linear up to 25 min and the

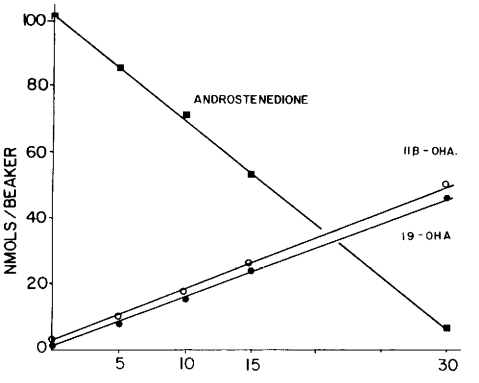

FIG. 1. Conversion of androstenedione (A) to 11β-hydroxyandrostenedione (11β-OH A) and 19-hydroxyandrostenedione (19-OH A) by gerbil adrenal homogenate incubated in the presence of 10 m*M* isocitrate.

two products were formed at nearly identical rates. In these experiments the rates of hydroxylation of A in the presence of isocitrate were up to 75% greater than those found with NADPH +10 m*M* Ca^{2+}. When malate was used to support the conversion, virtually no hydroxylated products were obtained after a 30-min reaction. Succinate supported hydroxylation to an extent similar to that seen with NADPH + Ca^{2+}.

A second series of experiments was undertaken to compare the ability of gerbil adrenal homogenate and subcellular fractions to hydroxylate androstenedione (Fig. 2). The highest activity for the conversion of A to both 19-OH A and 11β-OH A occurred in the mitochondrial fraction incubated in the presence of either isocitrate or NADPH + Ca^{2+} (Fig. 2). Though the formation of both hydroxylated products was found with other subcellular fractions, the activities were below that found with mitochondria. The ability of calcium ions to stimulate NADPH supported 19- and 11β-hydroxylation reactions was particularly evident in adrenal mitochondria and also occurred with adrenal homogenate and the nuclear fraction. Other experiments (data not shown) were carried out using 2 m*M* NADPH. Incubation of that concentration of NADPH with the 15,000*g* pellet and steroid substrate yielded barely detectable quantities of either hydroxylated product. With homogenate or other adrenal subcellular fractions the use of 2 m*M* NADPH failed to produce a significant effect over that seen using 1 m*M* cofactor. With the microsomal fraction or cytosol both 11β- and 19-OH A were formed in the presence of NADPH alone but the formation of products was not enhanced by the presence of Ca^{2+} (Fig. 2). There was, however, some difference in the ratio of products formed among the various fractions incubated with steroid substrate.

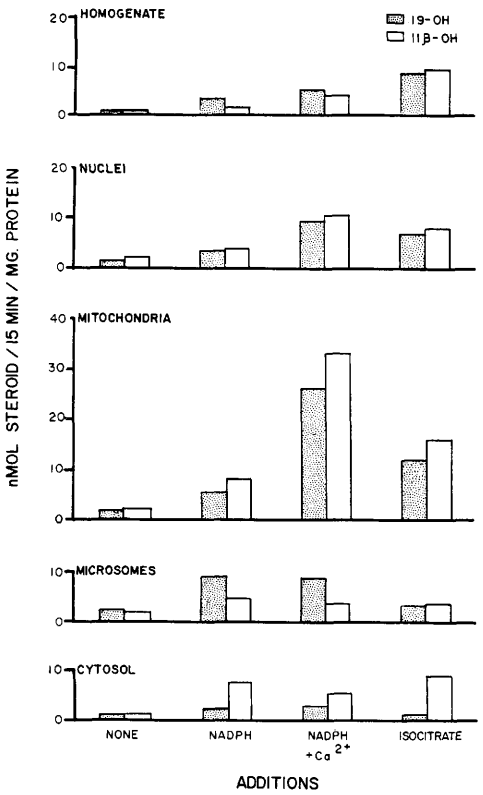

FIG. 2. The conversion of androstenedione (A) to 19-hydroxyandrostenedione and 11β-hydroxyandrostenedione by gerbil adrenal homogenate and subcellular fractions. Fractions incubated with 90 μM A with either 1 m*M* NADPH in the absence of presence of 10 m*M* Ca^{2+} or 10 m*M* isocitrate as a source of reducing equivalents.

With maximum stimulation of the hydroxylation conversions in adrenal homogenate or nuclear or mitochondrial fraction the amount of 19-OH formed was generally equal to or slightly less than that of the 11β-OH compound. Though lesser amounts of hydroxylated steroids were formed in the microsomal or cytosolic fractions, there was a difference in the ratio of the products. Of the small amount of steroids produced, the ratio of 19-OH A to 11β-OH A formed was higher in the microsomes than in the cytosol (Fig. 2).

When adrenal homogenates were incubated with NADPH alone under conditions wherein relatively little A was converted to 19-OH A or 11β-OH A (as in Fig. 2) we found evidence for two other compounds formed in the reaction. One component was subsequently found to have a mobility identical to testosterone (T) and the other to have a mobility identical to 19-hydroxytestosterone (19-OH T). While these components were detected in incubations of adrenal homogenate with NADPH, neither was present when homogenate was incu-

bated with isocitrate or succinate. After subcellular fractionation, the activity involved in the conversion of A to the component with the higher TLC mobility (T) appeared in the cytosolic fraction. The microsomal fraction retained the slight ability to convert A to what was identified as 19-OH T. The conversion of A to T was not evident in the nuclear, mitochondrial, or 15,000*g* fraction.

Because of interest in these latter observations, a study was conducted on the ability of the cytosol of the gerbil adrenal gland to interconvert A and T. The presence of a C-17 steroid oxidoreductase is indicated by the ability of the cytosol to interconvert A and T in the presence of the appropriate NADP cofactor (Fig. 3).

In subsequent experiments, gerbil adrenal homogenate and subcellular fractions were incubated with testosterone as substrate with results similar to those observed with A as steroid precursor. The highest specific activity for the conversion of T to 19-OH T and 11β-OH T was found in the mitochondrial fraction in the presence of isocitrate, succinate, or NADPH + Ca^{2+}. The formation of hydroxylated products by gerbil adrenal mitochondria is summarized in Table I; T or deoxycorticosterone (DOC) served as substrate. The experiments with DOC were conducted without using radioactive tracer; extracted steroid products were subjected to TLC separation adjacent to known standards including corticosterone (B) and 19-hydroxy DOC. The unknown samples were extracted from the TLC plates and their concentrations were determined by ultraviolet absorption at 240 nm. The highest conversion of precursor to either product occurred in the presence of NADPH + Ca^{2+} or with isocitrate.

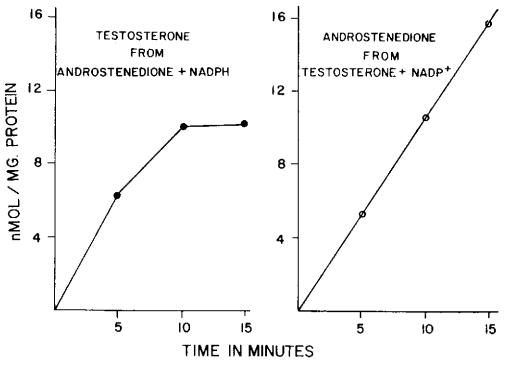

FIG. 3. Steroid C-17 oxidoreductase activity in gerbil adrenal cytosol was incubated with androstenedione + 1 m*M* NADPH (left) or with testosterone + 1 m*M* NADP (right).

TABLE I. CONVERSION OF TESTOSTERONE (T) OR DEOXYCORTICOSTERONE (DOC) TO 11β- AND 19-HYDROXYLATED PRODUCTS BY GERBIL ADRENAL MITOCHONDRIA

Incubation conditions, mitochondria plus	Products (nmole/beaker/15 min)[a]	
	19-OH T	11β-OH T
Testosterone	2	2
T + NADPH	6.6	8.7
T + NADPH + Ca^{2+}	16.9	21.2
T + isocitrate	11.5	18.1
T + succinate	6.6	7.8
	19-OH DOC	B
DOC + NADPH	8.2	10.2
DOC + NADPH + Ca^{2+}	17.2	19.6
DOC + isocitrate	24.0	18.3

[a] Beaker contained 180 nmole of steroid precursos plus 1 m*M* NADPH with or without 10 m*M* Ca^{2+} or 10 m*M* isocitrate or succinate.

Discussion. Our results indicate that the ability of the adrenal gland of the Mongolian gerbil to 19-hydroxylate steroid substrates is associated chiefly with enzymatic activity of the adrenal mitochondria. The mitochondrial fraction showed the highest activity for the formation of both 11β- and 19-OH steroid products and responded to various sources of reducing equivalents in a manner similar to that of rat adrenal mitochondria. Calcium ions have been shown to effect a great stimulation of NADPH-supported corticosteroidogenesis by rat adrenal mitochondria *in vitro* (14, 16, 17). That effect is, in part, related to Ca^{2+} ion-induced mitochondrial swelling leading to increased permeability to NADPH (14, 16). Similar effects appear to apply to the present experiments, since in gerbil adrenal homogenate and mitochondria, additions of Ca^{2+} with NADPH elevated the rates of both 11β- and 19-hydroxylation reactions. Substrates associated with the tricarboxylic acid cycle, permeable to the mitochondrial membrane, are also known to provide re-

ducing equivalents for mitochondrial steroid hydroxylations *in vitro* (see 14, 17, 18). In incubations of gerbil adrenal homogenate and mitochondria, isocitrate, and to a lesser extent succinate, both supported the formation of 11β- and 19-OH products (Figs. 1 and 2; Table I). These two steroid products were the only ones detected when gerbil adrenal homogenates or mitochondria were incubated with isocitrate; and both products were formed at nearly equal rates (Fig. 1). The 19-hydroxylase activity of the gerbil adrenal gland appears to be principally a mitochondrial enzyme coexisting there with the 11β-hydroxylase. The detection of the 19-hydroxylation activity in other adrenal subcellular fractions probably represents a redistribution of mitochondrial enzyme(s). In related experiments, gerbil adrenal mitochondria prepared by identical procedures were used to study respiration using the Clark oxygen electrode (McCarthy and Marshall, unpublished observations). The results of those studies included evidence that the mitochondria were coupled and that succinate-stimulated oxygen consumption was sensitive to antimycin A and cyanide.

It is evident that some of the mitochondria cosedimented with the nuclear and cell debris fraction. In this fraction, oxidizable substrate- as well as NADPH + Ca^{2+}-supported 19- and 11β-hydroxylations are evident. However, whether expressed on the basis of milligrams of protein or equivalent of wet weight, the extent of transformation of A was much less in the nuclear than in the mitochondrial fraction. The presence of little to no 11β- or 19-hydroxylation activity in the 15,000*g* pellet indicates the absence of intact mitochondria in that fraction. Consequently, intact mitochondria should be absent from the microsomal fraction and cytosol which are harvested after the 15,000*g* fraction. Therefore, the likely explanation for the steroid hydroxylase activity in the cytosol appears to be that resulting from release of enzymes from the mitochondria. This suggestion is supported by the fact that even mild homogenization of rat adrenal glands has been shown to release the mitochondrial cholesterol side chain cleavage activity to the cytosol (19). A similar release could be expected to account for the presence of relatively small amounts of 11β- and 19-hydroxylation activities in the cytosol and microsomal fractions. There was, however, some indication of a difference in distribution of the two hydroxylase activities between the cytosol and microsomal fraction. The ratio of 19- to 11β-hydroxylated products formed was greater in the microsomal fraction than in the cytosol. Whether this variation represents a differential redistribution of the two hydroxylase activities released from the mitochondria and/or the presence of some inherent microsomal 19-hydroxylase activity cannot be answered at this time.

The presence of steroid C-17 oxidoreductase in gerbil adrenal glands was indicated by the observation of trace amounts of testosterone derivatives found in homogenates incubated with androstenedione. The enzyme activity was found in the cytosol, where, with the appropriate NADP cofactor, A and T were interconverted at near equal rates (Fig. 3).

The efficiency with which isocitrate and succinate support steroid hydroxylations in the gerbil adrenal mitochondria is similar to that reported for the rat. The utilization of such oxidizable substrates to support 11β-hydroxylation in adrenal mitochondria has been shown to depend on the generation of intramitochondrial NADPH (14, 17, 20, 21). Thus succinate oxidation supports relatively low rates of mitochondrial hydroxylation because of the energetics involved in the reverse flow of electrons to generate NADPH (see 17). In contrast, isocitrate stimulates high rates of 11β-hydroxylation in rat adrenal mitochondria where this substrate is oxidized by an NADP-linked isocitric dehydrogenase (19). In bovine adrenal mitochondria, 11β-hydroxylation is more effectively carried out in the presence of malate where intramitochondrial NADPH is generated via a malic enzyme pathway (18). This is in contrast to the gerbil adrenal homogenates and mitochondria where isocitrate was the most effective oxidizable substrate to provide reducing equivalents for both 11β- and 19-hydroxylation reactions but where malate was

virtually ineffective. Finally, based on the effects of Ca^{2+} and of oxidizable substrates, it can be concluded that in the gerbil adrenal gland, both the 19- and 11β-hydroxylase are present in the mitochondria. These two enzymes appear to have the same order of magnitude of activity as shown by their capacity to convert steroid substrates to equal amounts of the two hydroxylated products. Thus, the adrenal mitochondria of the gerbil can be considered as the site of synthesis of the high levels of 19-OH steroid circulating in the blood of this species (10).

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