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The 11β-Hydroxylation of Progesterone and Deoxycorticosterone by Rat Adrenal Mitochondria*

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With the technical assistance of William F. Robidoux and Elisabeth Shepard

ABSTRACT: A study was undertaken to evaluate the *in vitro* 11β-hydroxylation of progesterone and 11-deoxycorticosterone (DOC) by rat adrenal mitochondria. Incubations of DOC, progesterone, or both steroids were carried out with mitochondria in the presence of reduced triphosphopyridine nucleotide (TPNH) with or without calcium ions (Ca^{2+}). The effect of increasing Ca^{2+} concentration on the conversion of DOC into corticosterone was a biphasic one; slight stimulation of the reaction occurred with Ca^{2+} up to 55 μM and maximum 11β-hydroxylation was achieved between 1 and 22 mM. In the absence of Ca^{2+}, progesterone appeared to be somewhat more readily 11β-hydroxylated than DOC. At concentrations above 55 μM, the effect of Ca^{2+} on progesterone 11β-hydroxylation was similar to that for DOC. In the mixture of the two steroids, the presence of DOC markedly inhibited the 11β-hydroxylation of progesterone. At the higher concentrations of Ca^{2+}, progesterone slightly reduced the 11β-hydroxylation of DOC; however, at low Ca^{2+} or in the absence of Ca^{2+}, progesterone markedly enhanced the formation of corticosterone from DOC. Radioisotope studies indicated that the extra corticosterone formed from incubations of progesterone plus DOC did not arise from progesterone. Moreover, studies on mitochondrial swelling suggested that the slight additional swelling of the particles in the presence of progesterone plus DOC would not account for the enhanced corticosterone formation.

Incubation of 10–120 μg of DOC with 60 μg of progesterone revealed that corticosterone formation was suppressed only at the higher steroid concentrations. The reduction of DOC 11β-hydroxylation by progesterone may be related to total steroid concentration rather than to a specific effect of progesterone. Incubation of 20–60 μg of progesterone with 30 μg of DOC revealed a progressive inhibition of 11β-hydroxyprogesterone formation by DOC. Thus, while progesterone is 11β-hydroxylated, the normal steroid substrate, DOC, is more readily 11β-hydroxylated by rat adrenal mitochondria incubated with both steroids in the presence of Ca^{2+} and TPNH.

In some studies of steroid 11β-hydroxylation by rat adrenal mitochondria, incubations of the particulate fraction with progesterone have been undertaken to show the quality of the preparation. A lack of microsomal contamination in the mitochondrial preparation was shown by the finding of 11β-hydroxyprogesterone[1] rather than 11-deoxycorticosterone (DOC) following incubations of the particulate fraction with progesterone (Péron *et al.*, 1964a,b, 1965a,b; Roberts *et al.*, 1964, 1965). It has been established that the 11β-hydroxylation of DOC by rat adrenal mitochondria in the presence of reduced triphosphopyridine nucleotide (TPNH, NAD PH) requires the presence of calcium ion (Ca^{2+}) (Péron *et al.*, 1965a,b, 1966).

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† Recipient of Research Career Award AM-K3-19-190 National Institute of Arthritis and Metabolic Diseases.

[1] The systematic chemical names of substances for which trivial names are used on this report are the following: 11-deoxycorticosterone (DOC), 21-hydroxy-4-pregnen-3,20-dione; 18-hydroxy-11-deoxycorticosterone (18-OHDOC), 18,21-dihydroxy-4-pregnen-3,20-dione; progesterone, 4-pregnen-3,20-dione; 11β-hydroxyprogesterone, 11β-hydroxy-4-pregnen-3,20α-dione; corticosterone, 11β-21-dihydroxy-4-pregnen-3,20-dione; TPNH, reduced triphosphopyridine nucleotide; ACTH, adrenocorticotrophin.

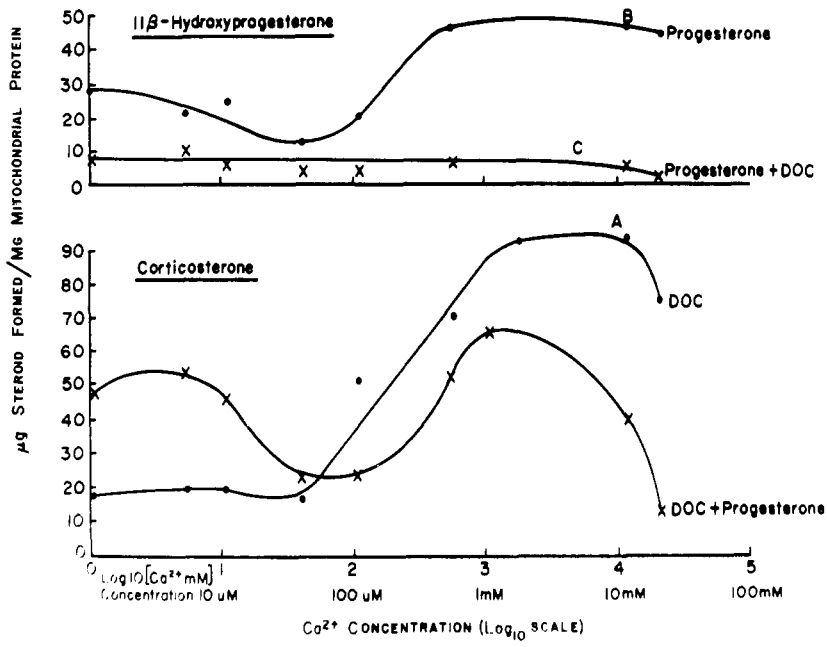


FIGURE 1: Effect of Ca^{2+} concentrations of the *in vitro* 11β-hydroxylation of DOC and progesterone by rat adrenal mitochondria. Steroid substrate: DOC (60 μg) (curve A), progesterone (60 μg) (curve B), and DOC (60 μg) + progesterone (60 μg) (curves C and D). Mitochondria (0.46 mg of protein/beaker) were incubated with steroid substrate in 0.104 M $NaHCO_3$, with 800 μg of TPNH/beaker, to a final volume of 2.0 ml with 0.154 M KCl. Final Ca^{2+} concentrations are indicated on logarithmic scale in the figure. Incubation was carried out for 30 min at 37° under O_2–CO_2(95:5)

We undertook these studies to determine if the mitochondrial 11β-hydroxylation of progesterone showed a Ca^{2+} dependence similar to that for DOC (Birmingham *et al.*, 1953; Péron and Koritz, 1958; Péron *et al.*, 1964a, 1965a, 1966). Moreover, it was of interest to determine the effects of incubation of both steroids with the mitochondrial preparations. Since DOC appears to be the normal substrate for mitochondrial 11β-hydroxylation it seemed reasonable that the presence of progesterone might reduce the conversion of DOC into corticosterone. The latter view seemed reasonable in view of the early report by Brownie *et al.* (1954) that progesterone reduced the 11β-hydroxylase activity of ox adrenal mitochondria.

Materials

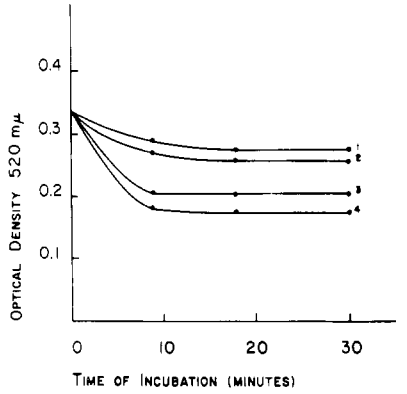
TPNH was obtained from Sigma Chemical Co., St. Louis, Mo. The tritriated progesterone, [1,2α-^{3}H]progesterone, was mixed with unlabeled progesterone to obtain a final activity of 4.79×10^6 dpm/60 μg of progesterone. The activity of [11β-hydroxy-4-^{14}C]progesterone used for isotope dilution analyses was 1.375×10^6 dpm/60 μg.

Methods

Adrenal glands were removed from groups of male Sprague–Dawley rats after decapitation. The adrenal glands were cleaned of adhering fat, weighed, and homogenized in cold 0.25 M sucrose. The mitochondrial fraction was obtained by the method described by Péron (1964a). The mitochondrial pellet (pellet 2, P-2) was resuspended in 0.154 M KCl and aliquots incubated in $NaHCO_3$ buffer, pH 7.4. Where present, Ca^{2+} was in the concentration range of 5 μM to 22 mM. The TPNH was added at 800 μg/beaker. The steroids, DOC or progesterone, were added in 0.01 or 0.02 ml of propylene glycol–absolute ethanol (1:1) to provide concentrations of substrate noted in results. The final volume in all beakers was brought to 2.00 ml with 0.154 M KCl. The final concentration of ethanol in all flasks was kept constant by adding the appropriate volume of propylene glycol–ethanol (1:1). In no case did the concentration of ethanol exceed 2%. Incubations were carried out for 30 min at 37° in a Dubnoff incubator under O_2–CO_2 (95:5). Products of reaction are expressed as micrograms per milligram of mitochondrial protein or as μg/2.0 ml (μg/2.0 ml or micrograms per beaker).

Analysis

After incubation, appropriate aliquots of the incubation media were taken and the corticosterone was determined by a modification (Moncloa *et al.*, 1959) of the sulfuric acid fluorescence technique (Silber *et al.*, 1958). The determination of 18-hydroxydeoxycorticosterone (18-OHDOC) was by a modification of the Porter–Silber reaction (Cortes *et al.*, 1963). The amount of [11β-hydroxy-1,2a-^{3}H]progesterone formed was determined by isotope dilution using additions of [11β-hydroxy-4-^{14}C]progesterone as a standard for the deter-



Incubation Condition with 800 μg TPNH	Products μg/tube B	11β-OH-Progesterone
1. DOC	8.98	–
2. DOC+Progesterone	17.39	8.86
3. DOC+Ca^{2+} 11mM	37.27	–
4. DOC+Progesterone +Ca^{2+} 11mM	24.96	1.06

FIGURE 2: Adrenal mitochondrial swelling response and 11β-hydroxylation during incubation with DOC and progesterone. Curve 1, DOC; 2, DOC + progesterone; 3, DOC + Ca^{2+} (11 mM); and 4, DOC + progesterone + Ca^{2+} (11 mM). Incubation conditions: Tris buffer, pH 7.3 (0.20 mM), nicotinamide (5.0 mM), NaCl (50 mM), Ca^{2+} final concentration as indicated, 800 μg of TPNH, 60 μg of DOC, or 60 μg of progesterone (or both steroids, 60 μg each) each added in 0.02 ml of equal volumes propylene glycol–ethanol; final volume brought to 1.0 ml with 0.154 M KCl. Samples, in glass-stoppered Coleman spectrophotometer tubes, were incubated at 37° and optical density readings (at 520 mμ) were taken at 0, 9, 18, and 30 min to measure mitochondrial swelling.

mination of recovery. The carbon-labeled compound was added to an aliquot for the incubation media which was then extracted with methylene chloride. The dried extract was taken up in ethanol and chromatographed on a thin layer of silica gel using an ethyl acetate–chloroform solvent system (McCarthy *et al.*, 1964; Quesenberry and Ungar, 1964). Measurements of ^{3}H and ^{14}C in samples removed from the chromatograms were made using a liquid scintillation counter (Packard Tri-Carb Model 314-DC). Determinations of the protein content of the mitochondrial preparation were carried out by the method of Lowry *et al.* (1951).

Results

The effect of Ca^{2+} concentration on the 11β-hydroxylation reactions is shown in Figure 1. The biphasic stimulation of the 11β-hydroxylation of DOC (curve A) is similar to that previously reported (Péron *et al.*, 1965a,b, 1966). A slight stimulation of the conversion of DOC into corticosterone was seen with Ca^{2+} levels up to 55 μM. In this study, maximum stimulation of the DOC reaction was seen with Ca^{2+} in the range of 1–22 mM.

In the absence of Ca^{2+}, somewhat more progesterone than DOC was 11β-hydroxylated (Figure 1, curve B). The addition of Ca^{2+} up to 55 μM to progesterone gradually repressed formation of 11β-hydroxyprogesterone. Higher levels of Ca^{2+}, above 55 μM, enhanced 11β-hydroxyprogesterone formation similar to that for DOC.

The addition of DOC to progesterone (in near-equimolar quantities) markedly repressed the 11β-hydroxylation of progesterone (Figure 1, curve C) regardless of Ca^{2+} concentration. Conversely, when progesterone was added to DOC (Figure 1, curve D) the formation of corticosterone was depressed at high concentrations of Ca^{2+} (above 55 μM) but enhanced at lower concentrations of Ca^{2+}. In Figure 1, the formation of corticosterone is shown to be increased in the presence of progesterone at Ca^{2+} concentrations 0–55 μM.

Further confirmation of the enhancement of corticosterone formation from DOC in the presence of progesterone is presented in Table I. As first noted in expt 1, the values for corticosterone produced by incubating DOC in the presence of progesterone were

TABLE I: Corticosterone Production from Deoxycorticosterone in the Presence or Absence of Progesterone.[a]

Expt	Ca^{2+} (μM)	Corticosterone Produced (μg/mg of mitochondrial protein) DOC	Proges-terone	DOC + Proges-terone
1	0	16.3	—	48.1
	5.5	20.8	—	55.9
	11.0	20.4	—	47.5
2	0	6.5	2.8	18.9
3	0	9.8	5.0	29.7
4	0	9.4	3.1	18.2

[a] Incubation conditions were the same as noted in Figure 1. The data in this table refer to the amount of corticosterone formed from incubations of rat adrenal mitochrondria with DOC or progesterone and DOC plus progesterone.

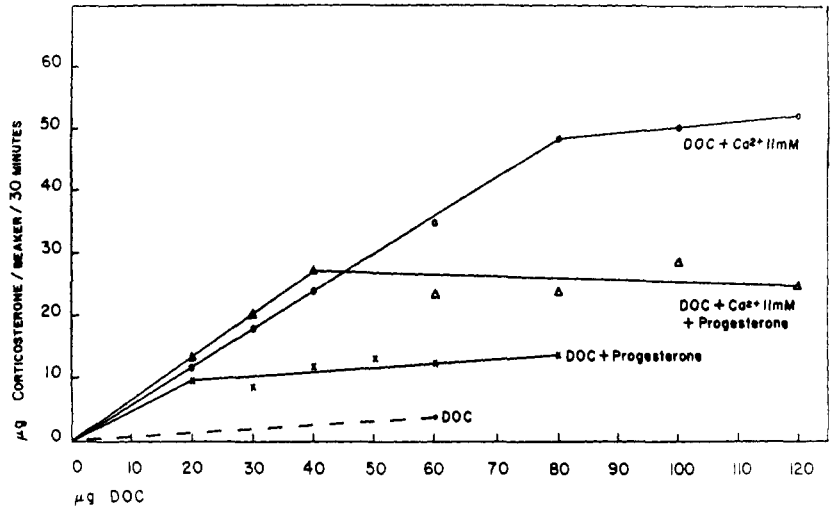


FIGURE 3: The effect of progesterone on the formation of corticosterone from various concentrations of DOC. Incubation conditions as indicated in Figure 1, except DOC present at concentrations noted in the figure; where present progesterone concentration was 60 μg, Ca^{2+} (11 mM).

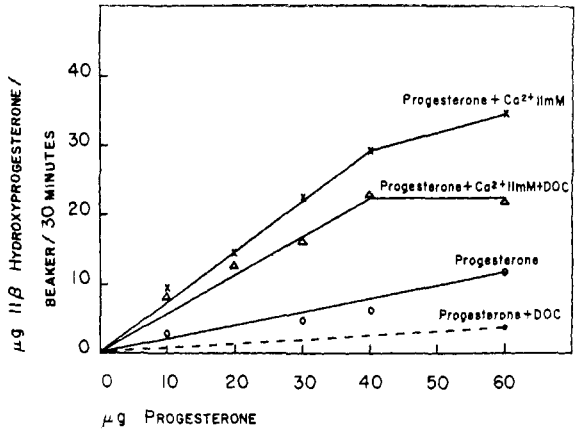


FIGURE 4: Influence of DOC on the production of 11β-hydroxyprogesterone from various concentrations of progesterone. Incubation conditions as indicated in Figure 1 except progesterone was incubated at concentration noted in the figure; where present, DOC concentration was 30 μg, Ca^{2+} (11 mM).

two to three times greater than found after incubation of DOC alone with rat adrenal mitochondria in the presence or absence of low levels of Ca^{2+}. In subsequent experiments, the fluorometric analysis of media after incubation of P-2 with progesterone alone indicated that the corticosterone produced from progesterone did not account for the increase in corticosterone formed in the mixture of the two steroid substrates. After the incubation of DOC and tritriated progesterone, samples of the media were extracted with methylene chloride and chromatographed on silica gel. Areas representing DOC, 11β-hydroxyprogesterone, progesterone, corticosterone, and 18-OHDOC were detected. When the tritium content of the spots representing corticosterone was determined, the results indicated that only 1.5% of tritriated progesterone was converted in corticosterone.

In an effort to explain the progesterone effect on the DOC reaction, a study of mitochondrial swelling was undertaken, since previous study indicated particle swelling was in part associated with the Ca^{2+} enhancement of 11β-hydroxylation (Péron *et al.*, 1965a,b). Thus, to test the enhancement of DOC hydroxylation by progesterone, adrenal mitochondria were incubated in a 20 mM Tris buffer (pH 7.4) with one or both steroids (60 μg each) in the presence of TPNH (800 μg). The swelling of mitochondria was measured spectrophotometrically (at 520 mμ) (Figure 2). In the absence of Ca^{2+}, the particle swelling in the presence of progesterone + DOC (or of progesterone alone), curve 2, was slightly greater than that with DOC alone (curve 1). However, the incubation of DOC with progesterone gave nearly a twofold increase in corticosterone production. The large degree of mitochondrial swelling was seen with the addition of Ca^{2+}, 11 mM (curves 3 and 4). In these cases, the Ca^{2+} induced a fourfold increase in corticosterone formation from DOC alone (curve 3) with a lesser amount formed from DOC in the presence of progesterone (curve 4).

The inhibitory effect of one steroid on the 11β-hydroxylation of the other was next studied by incubating a fixed quantity of one compound with various amounts of the other steroid. These studies were undertaken to determine if any relationship existed between steroid concentration and inhibition of the mitochondrial 11β-hydroxylation reaction. The results of the mitochondrial 11β-hydroxylation of DOC is shown in Figure 3. The production of corticosterone is illustrated for various concentrations of DOC with and without Ca^{2+} (11 mM) and/or progesterone (60 μg). In the absence of Ca^{2+}, a maximum of 5 μg of DOC was 11β-hydroxylated over the range of DOC tested (60 μg), while some 50 μg of corticosterone was produced in the

presence of 11 mM Ca^{2+} when 80 μg of DOC was used. Similar experiments carried out in the absence of Ca^{2+} but in the presence of progesterone led to an increase in corticosterone formation (8–11 μg). On the other hand, the mixture of Ca^{2+} and progesterone with DOC showed only a maximum corticosterone yield of 30 μg. Concentrations of DOC above 40 μg gave no additional amounts of corticosterone when incubated with mitochondria in the presence of 60 μg of progesterone.

The influence of DOC on the progesterone conversion was next studied. In these studies, 30 μg of DOC was added to flasks containing up to 60 μg of progesterone (Figure 4). In the absence of Ca^{2+}, the linear increase in 11β-hydroxyprogesterone formation reached a maximum of 10 μg from 60 μg of substrate. The addition of DOC inhibited the formation of 11β-hydroxyprogesterone while Ca^{2+} induced the marked stimulation of this 11β-hydroxylation of the various levels of progesterone. In the presence of Ca^{2+}, addition of the DOC progressively inhibited the 11β-hydroxylation of the levels of progesterone incubated up to 40 μg.

Table II presents the results of several series of studies on the incubations of DOC, progesterone, and both steroids. Again, it may be noted, that in the absence of Ca^{2+}, progesterone enhanced the conversion of DOC into corticosterone. At high levels of Ca^{2+}, large concentrations of progesterone were required to significantly depress the corticosterone formation. However, even the low concentrations of DOC (20–30 μg) reduced the 11β-hydroxylation of progesterone without depression of corticosterone formation. It can also be seen that the effects of the incubation conditions on the 18-OHDOC production were those that generally followed the pattern of corticosterone formation.

TABLE II: *In Vitro* Production of Corticosterone, 18-OHDOC, and 11β-Hydroxyprogesterone by Adrenal Mitochondria Incubated with Various Combinations of DOC and Progesterone.[a]

Substrate (μg)		Steroid Production (μg/beaker)		
DOC	Progesterone	Corticosterone	18-OHDOC	11β-OH-Progesterone
60	*b*	3.7	3.1	—
—	60[b]	1.8	—	12.3
60	60[b]	11.1	5.7	2.9
20	—	12.0	7.5	—
30	—	18.2	9.9	—
40	—	24.1	15.1	—
50	—	27.9	—	—
60	—	35.3	20.0	—
80	—	45.3	22.4	—
—	10	1.7	1.6	9.6
—	20	1.6	2.9	15.5
—	30	1.8	—	23.8
—	40	1.8	4.0	28.6
—	60	1.8	0.8	37.0
20	60	12.6	15.2	30.9
30	60	18.9	15.4	21.2
40	60	27.4	14.7	21.6
50	60	33.7	21.3	10.9
60	60	40.7	25.1	8.5
80	60	39.3	2 .8	6.5
30	10	20.0	12.5	9.4
30	20	21.3	11.3	13.0
30	30	19.3	—	16.7
30	40	20.0	10.0	24.0
30	60	20.0	12.3	21.6

[a] Incubation were those indicated in Figure 1. Mitochondrial protein is 0.65 mg/beaker. [b] Incubated in the absence of Ca^{2+}; all other data obtained from incubations in the presence of Ca^{2+} (11 mM).

Discussion

The chief objective of this study was to evaluate the role of Ca^{2+} in the 11β-hydroxylation of progesterone in the presence of TPNH by rat adrenal mitochondria. In this study the biphasic curve for the effect of Ca^{2+} on corticosterone formation (Figure 1) was found and is similar to that reported previously by Péron and Koritz (1958) for rat adrenal gland homogenates. Also, Figure 1 demonstrates that while high Ca^{2+} levels had a marked effect in stimulating the 11β-hydroxylation of progesterone, the reaction did proceed in significant amounts in the absence of Ca^{2+}. Thus, in the absence of Ca^{2+} more progesterone than DOC was 11β-hydroxylated by the adrenal mitochondria when the individual steroids were incubated (Table II).

Brownie *et al.* (1954) noted that progesterone inhibited the 11β-hydroxylase activity of ox adrenal mitochondria with a reduction in some steps in oxidative metabolism and oxygen consumption. Roberts *et al.* (1965) found that the basal rates of 11β-hydroxylation of progesterone and DOC by rat adrenal mitochondria were similar. Our data are in agreement with the reports of Péron *et al.* (1965a,b), Roberts *et al.* (1965), and McCarthy *et al.* (1966) that progesterone is readily 11β-hydroxylated by rat adrenal mitochondria. There is a difference between our data and those of Brownie *et al.* (1954) on the 11β-hydroxylation of progesterone. However, in addition to difference in species and ratio of steroid and protein employed, Brownie *et al.* (1954) used metabolic substrates to support the 11β-hydroxylation reaction while our studies were undertaken with exogenous TPNH. In one experiment, undertaken in the Tris buffer, we did note a decrease in 11β-hydroxyprogesterone formation in the presence of oxidizable substrate. The 11β-hydroxylated products from incubation of DOC and progesterone, respectively, were 24.3 and 12.2 μg with isocitrate (10 mM) and 7.4 and 4.4 μg with succinate (10 mM). This one experiment, however, does not indicate that progesterone in rat adrenal mitochondria is capable of depressing oxidative processes as noted by Brownie *et al.* (1954) for ox adrenal mitochondria. Moreover, since the incubations

were carried out in the presence of Ca^{2+} (11 mM), with resultant mitochondrial swelling, exogenous TPNH would support the 11β-hydroxylation and mask an effect of depressed substrate oxidation (Guerra *et al.*, 1966; Péron *et al.*, 1966).

The significant findings of these incubation studies, undertaken to evaluate the effect of progesterone and DOC on the 11β-hydroxylation, indicated that (a) progesterone stimulated the conversion DOC into corticosterone in the absence of Ca^{2+}, (b) there was a suppression of the 11β-hydroxylation of DOC by high concentrations of progesterone, and (c) a general suppression of 11β-hydroxylation of progesterone by DOC in the presence or absence of Ca^{2+} occurred. Both observations a and c are of interest and might examplify the physiological mechanism of control whereby progesterone is channeled into the proper pathway leading to the steroid intermediate (DOC) which is eventually transformed to the terminal secretory product of the rat adrenal gland (corticosterone). At present we do not know whether competitive inhibition of the 11β-hydroxylase enzyme by progesterone or DOC has played some role in our incubation studies (see below).

Several workers have utilized the incubation of progesterone with mitochondria to indicate the lack of contamination with microsomes and to study mechanisms of 11β-hydroxylation (Péron *et al.*, 1965a,b; Roberts *et al.*, 1964, 1965). Should the mitochondria be contaminated with microsomes (containing the 21-hydroxylase), progesterone would presumably be converted into DOC and then into corticosterone. Our results of this study support this test for microsomal contamination, for as was seen, the incubation of progesterone gave rise to 11β-hydroxyprogesterone, with very little corticosterone formed (Tables I and II). On the other hand, incubations of DOC and progesterone together gave rise to more corticosterone than did incubation of DOC alone in the absence of Ca^{2+} (Figure 1, Tables I and II). The isotope analysis revealed that after incubation of DOC with radioactive progesterone, a maximum of 1.5% of the tritriated progesterone was converted into corticosterone. Thus, it can be concluded that the extra corticosterone produced from incubation of these two steroids arose from the 11β-hydroxylation of DOC rather than from progesterone. It can be said, therefore, that progesterone stimulated 11β-hydroxylation of DOC. Mitochondrial swelling could not explain the progesterone stimulation of the DOC conversion. Péron and co-workers (1965a,b, 1966) demonstrated that part of the Ca^{2+} requirement for 11β-hydroxylation of DOC in the presence of TPNH was related to mitochondrial swelling. If progesterone had induced mitochondrial swelling, this effect might have accounted for the extra corticosterone formed. However, the swelling induced by progesterone + DOC was only slightly greater than that with DOC alone and as seen in Figure 2, the corticosterone formation was increased nearly twofold. In the same experiment, incubation of DOC + 110 μM Ca^{2+} gave nearly the same swelling (final optical density 0.22) as that for progesterone + DOC (optical density 0.26, curve 2, Figure 2) yet the corticosterone formation with this degree of mitochondrial swelling (by Ca^{2+}) was less than with the mixture of steroids (13.0 *vs.* 17.4 μg). It is also of interest to note that addition of progesterone to DOC + Ca^{2+} (110 μM) induced a mitochondrial swelling (final optical density 0.21) with an increased corticosterone formation over that seen with DOC + Ca^{2+} (110 μM) (19.3 *vs.* 13.0 μg, respectively). Péron *et al.* (1965b) demonstrated that maximum mitochondrial swelling induced by agents other than Ca^{2+} failed to stimulate maximum corticosterone formation. Thus, it appears unlikely that the slight additional swelling produced by adding progesterone to DOC would account for the enhanced corticosterone formation. In addition, adding progesterone to DOC + Ca^{2+} (110 μM), as noted above, increased, corticosterone formation without excessive swelling.

When the two steroids were incubated with rat adrenal mitochondria in the presence of Ca^{2+} and TPNH some degree of reciprocal inhibition of 11β-hydroxylation was noted. The presence of 60 μg of DOC effectively reduced the 11β-hydroxylation of progesterone, regardless of Ca^{2+} concentrations (Figure 1). When the concentration of progesterone was varied and incubated with 30 μg of DOC (Figure 2), the effect of DOC on inhibition of 11β-hydroxyprogesterone formation is suggestive of that of a competitive inhibitor. Analysis of the data suggests this type of inhibition might occur but studies with additional concentrations of DOC are required before this suggestion can be confirmed.

Several studies by other investigators have been carried out to study the effect of progesterone on *in vitro* steroid conversions by adrenal tissues and on *in vivo* corticosteroidogenesis. These studies indicate inhibitory effects of progesterone but not necessarily on the DOC hydroxylation. Kowal *et al.* (1964) reported an inhibition by progesterone on the Δ^5-3β-hydroxy steroid isomerization by an adrenal preparation. Recently, Villee (1966) reported the depressed activity of 3β-hydroxy steroid dehydrogenase in fetal adrenal tissue preincubated with progesterone. Villee suggests a possible *in vivo* role for progesterone indicating this steroid could influence enzymes in the adrenal gland of the fetus. An *in vivo* suppression of adrenal corticosterone release was reported by Singer *et al.* (1963) to follow the administration of progesterone to rats. Steinetz *et al.* (1965) found that after administration of progesterone (and other progestagenic steroids) for 14 days to male rats, there was a significant depression of the corticosterone response after ACTH injection. Lastly, Makoff *et al.* (1964) have found that addition of rat serum at the beginning of *in vitro* incubations with rat adrenal homogenate + progesterone markedly enhanced the formation of DOC and completely obliterated the production of 11β-hydroxyprogesterone. In the present study, the ability of progesterone to depress corticosterone formation from high levels of DOC may not involve any of the above-cited mechanisms and only represent an inhibitory effect because of total steroid concentration rather than a specific effect of

progesterone.

Thus, in the experiment shown in Figure 3, 60 μg of progesterone had no effect on reducing corticosterone formation until the DOC concentration exceeded 40 μg. In the studies noted in Table II, progesterone suppression of this reaction was apparent only with 80 μg of DOC. In these two studies, there was a variation in the concentration of mitochondrial protein also and in the experiment reported in Figure 3, protein content of each beaker was 0.38 mg, while for the studies noted in Table II, 0.68 mg of mitochondrial protein was in each beaker. Péron and McCarthy (1966) have shown that the concentration of protein per incubation beaker influences the amount of steroid converted, for it was found that under identical incubation conditions the production of corticosterone (micrograms per milligram of protein) did vary with the amount of protein incubated. Thus, in this study, if the effect of progesterone on the hydroxylation of DOC is related to total steroid concentration and possible excess substrate inhibition, then the reduction may be more likely to occur with smaller amounts of mitochondria (as shown in Figure 3 *vs.* Table II). Nevertheless, it seems unlikely that this effect of progesterone on DOC formation would be of likely significance in *in vivo* corticosterone secretion, as in the works noted previously (Singer *et al.*, 1963; Steinetz *et al.*, 1965).

Finally, the data derived from this study do indicate that maximum 11β-hydroxylation of progesterone by rat adrenal mitochondria supported by TPNH does show a Ca^{2+} dependence similar to that for DOC. However, unlike the reaction for DOC, in the absence of Ca^{2+} progesterone is somewhat more readily 11β-hydroxylated. When the two steroids are incubated together with the mitochondria, DOC appears to be hydroxylated more readily than progesterone. DOC generally reduced the hydroxylation of progesterone by what may be a competitive inhibition. The Ca^{2+}-stimulated hydroxylation of DOC that is suppressed by progesterone may reflect an influence of total steroid concentration rather than a specific effect of progesterone. In the absence of Ca^{2+}, progesterone was found to enhance the mitochondrial 11β-hydroxylation of DOC. This effect of progesterone is an intriguing one for the extra corticosterone produced does not appear to originate from progesterone or to be a response from extra mitochondrial swelling. However, while progesterone can be 11β-hydroxylated by rat adrenal mitochondria, 11β-hydroxylation reaction appears to favor the steroid substrate DOC.

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