Further Studies on the 11β-Hydroxylation of Deoxycorticosterone and Progesterone by Rat Adrenal Mitochondria*

Burton V. Caldwell,† Fernand G. Péron, and John L. McCarthy‡

With the Technical Assistance of William F. Robidoux, Jr., and Linda Martel

ABSTRACT: Rat adrenal mitochondria utilized oxidizable substrates (isocitrate, α-ketoglutarate, and succinate) to provide reducing equivalents (intramitochondrial reduced tri- and diphosphopyridine nucleotides, TPNH and DPNH) for 11β-hydroxylation of 11-deoxycorticosterone (DOC) and progesterone. Exogenous TPNH and low concentrations of Ca^{2+} (5.5 μM) were also utilized in this respect. Combining DOC and progesterone resulted in a significantly lowered DOC to corticosterone conversion when oxidizable substrates supported the hydroxylations. However, progesterone stimulated this conversion when exogenous reduced cofactor provided the reducing equivalents. DOC markedly inhibited the formation of 11β-OH-progesterone regardless of the additive. Mitochondrial oxygen utilization was decreased by adding progesterone to oxygraph cells containing DOC and isocitrate, but increased when progesterone was added to DOC if the mitochondria were incubated with TPNH and Ca^{2+}. DOC was more readily hydroxylated than progesterone and mitochondrial oxygen uptake was consistantly greater with DOC, regardless of the reducing agent. Pregnenolone added as substitute for either DOC or progesterone showed no influence on any of the above-mentioned parameters. The 103,000*g* supernatant fraction of adrenal gland homogenates from several species of animals (rat, guinea pig, and bovine) supported 11β-hydroxylation in a manner similar to that of the oxidizable substrates, inasmuch as progesterone inhibited DOC to corticosterone conversion with this supernatant supplying the reducing equivalents. These data suggest that the mechanism generating reducing equivalents may influence the direction and extent of steroid hydroxylation in the rat adrenal mitochondria.

A recent discussion of the effects of progesterone on the metabolic processes of adrenal mitochondria (McCarthy and Péron, 1967) provides a basis for examining the activity of the 11β-hydroxylase enzyme system, comparing the utilization of reducing equivalents produced by oxidizable substrates, exogenous TPNH[1] and Ca^{2+}, and supernatants from several species of animals. Previous suggestions that there may be a substrate specificity as related to mitochondrial steroid 11β-hydroxylations have fallen short of providing substantial evidence in support of this contention. A preliminary report (Grant, 1962) indicating that there may be two distinct 11β-hydroxylase enzyme systems in adrenal glands, one for progesterone and another for hydroxylating the normal steroid substrate 11-deoxycorticosterone (DOC), was based on the difference in activity of these two proposed enzymes in the presence of the inhibitor SU 4885 (2-methyl-1,2-bis(3-pyridyl)propan-1-one, Metopyrone). This work, however, has not been substantiated beyond the preliminary stages.

This production of 11β-hydroxyprogesterone as an intermediate in the biosynthesis of adrenal steroids has been under intensive study since the work of Brownie *et al.* (1954). They suggested that, although this conversion might not normally be of major importance, in some pathological conditions the 11β-hydroxylation of progesterone may be considerably increased, as substantiated by Eichorn and Hechter (1958) in the case of an aldosterone secreting tumor, and alluded to by Grant (1962) as work done by Brode in his laboratory. McCarthy and Péron (1967) showed clearly that the Ca^{2+} stimulation of progesterone 11β-hydroxylation by rat adrenal mitochondria in the presence of exogenous TPNH was biphasic, similar to the findings of several investigators for the DOC to cortico-

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† A.R.C. Unit for Reproductive Physiology and Biochemistry, Cambridge, England.

‡ Associate Professor, Department of Biology, Southern Methodist University, Dallas, Texas.

[1] The systematic chemical names of substances for which trivial names are used on this report are the following: TPN, oxidized triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; 11-deoxycorticosterone (DOC), 21-hydroxy-4-pregnen-3,20-dione; 18-hydroxy-11-deoxycorticosterone (18-OH DOC), 18,21-dihydroxy-4-pregnen-3,20-dione; corticosterone (B), 11β-21-dihydroxy-4-pregnen-3,20-dione; progesterone, 4-pregnen-3,20-dione; 11β-hydroxyprogesterone, 11β-hydroxy-4-pregnen-3,20-dione; pregnenolone, 3β-hydroxy-5-pregnen-20-one.

sterone conversion (Koritz and Péron, 1959; Péron *et al.*, 1966). Of more interest, however, was the observation that without or with low Ca^{2+} concentrations (5.5 μM final concentration) significant stimulation of the DOC to corticosterone resulted when progesterone was added in near-equimolar concentrations to the incubation medium. This increased corticosterone production was not a product of progesterone to corticosterone which would be dependent upon microsal contamination of the mitochondrial pellet to accomplish the hydroxylation reaction at carbon position 21. At higher concentrations of Ca^{2+} (0.5–10 mM) the stimulation was not observed and gave way to a significant inhibition of corticosterone formation with the same amounts of progesterone added.

The above findings, coupled with the early report of Brownie *et al.* (1954), noting that progesterone inhibited the 11β-hydroxylase activity of ox adrenal mitochondria with a reduction in some steps in oxidative metabolism when oxidizable substrates were used to support the hydroxylations, led us to the present investigation. Our purpose was to examine mitochondrial hydroxylation of DOC and progesterone in various combinations, and to compare the 11β-hydroxylation of these steroid substrates using exogenous cofactor (TPNH and Ca^{2+}), oxidizable substrates, and high-speed adrenal supernatant fraction from several animal species as agents for providing reducing equivalents. Also, a study of mitochondrial oxygen uptake during these conversions, and with all of the various steroid combinations with different reducing agents, was undertaken. The oxygen-uptake studies were carried out to indicate the possible role played by progesterone in inhibiting the P-450 chain which is involved in steroid hydroxylation reactions (Omura *et al.*, 1965, 1966; Harding *et al.*, 1965; Simpson and Boyd, 1967; Cooper *et al.*, 1965).

Materials and Methods

The materials and methods used in this investigation are essentially those reported by McCarthy and Péron (1967) with the following additions. The mitochondrial pellet (Pellet-2, P-2) was prepared as described by Péron *et al.* (1965a) and resuspended in a mixture of sucrose (250 mM), Tris buffer (15 mM), and nicotinamide (20 mM) from which aliquots were taken for the incubations. All incubations were carried out in 3-ml, stoppered, matched Coleman tubes containing Tris buffer (15 mM), nicotinamide (50 mM), NaCl (15 mM), KCl (15 mM), $MgCl_2$ (10 mM), bovine serum albumin (0.1%), and brought to a final volume of 1 ml/tube (including all other additives) with 0.25 M sucrose at a pH of 7.4.

Steroids were added in a propylene glycol–absolute ethanol mixture (1:1) with final ethanol concentrations kept constant below 2% in the incubation medium. The incubations were carried out at 37° for 30 min unless otherwise specified, and the tubes were inverted for mixing purposes at 5-min intervals. At the end of the incubation, the contents of the tubes were frozen immediately by placing the tubes in an ethanol and Dry-Ice mixture.

Mitochondrial oxygen uptake was measured polarographically using a vibrating platinum electrode of an oxygraph (Model KM, Gilson Medical Electronics, Wis.). Recordings at 25 and 37° were taken by circulating water at the desired temperature through the water-jacketed cell surrounding the incubation chamber.

The final concentration of all oxidizable substrates (*e.g.*, isocitrate) was 10 mM while TPNH was added at 800 μg/tube. Where present, the final Ca^{2+} concentration was 5.5 μM. The high-speed supernatant fractions (103,000*g* for 60 min) of adrenal gland homogenates from several species of animals were obtained by the fractionation procedure outlined previously by Péron *et al.* (1965b). Where used, these were added just prior to the incubations (0.20 ml; about 1.3 mg of protein). The original homogenate was prepared by homogenizing approximately 1900 mg wet wt of adrenal gland tissue in 7.4 ml of homogenizing medium.

The steroid analyses were as described by McCarthy and Péron (1967). Protein determinations were carried out by the method of Lowry *et al.* (1951).

Results

The effect of added progesterone on the 11β-hydroxylation of DOC to corticosterone (B) is clearly inhibitory when the oxidizable substrates isocitrate, α-ketoglutarate, and succinate are used to produce intramitochondrial TPNH (Table I). However, as previously shown by McCarthy and Péron (1967), in the presence of exogenous TPNH (800 μg) and low Ca^{2+} concentrations, the effect of added progesterone is to stimulate this conversion (Table II). Results obtained with the high-speed supernatant fraction from the pellet-4 centrifugation indicate that the reducing power of this cell fraction is similar to that of the oxidizable substrates since corticosterone production is greatly reduced in the presence of progesterone (Table II).

Because of unavoidable inherent variations in steroid 11β-hydroxylating capacity, as a result of preparing mitochondria from different groups of animals and on different days and used for different experiments, and the fact that the mitochondrial protein concentrations used varied from experiment to experiment (0.68–0.98 mg of P-2 protein/tube), the per cent change in the amount of DOC transformed into B in the different experiments was used for reporting the inhibitory effects of progesterone on this reaction (last column, Table I). As can be seen, the per cent change was always a substantial negative value when oxidizable substrates like isocitrate or supernatant fraction from the several species of animals were used in the incubations. The per cent change in values was positive when exogenous TPNH and Ca^{2+} were added. Thus, the effect of progesterone on DOC to B conversion in the different experiments is more readily seen when the values are reported in the above manner, than if they were reported on a basis of comparison of the absolute corticosterone production only.

TABLE I: Steroid 11β-Hydroxylation by Rat Adrenal Mitochondria Incubated with Deoxycorticosterone, Progesterone, and Pregnenolone in the Presence of Various Oxidizable Substrates.[a]

	Steroid Added (μg/tube)			Steroid Formed (μg/tube)		
Additions	DOC	Progesterone	Pregnenolone	Corticosterone	11β-OH Progesterone	% Change[b]
Isocit	120			66.5		
		120		2.6	51.8	
			120	0.6		
	120	120		54.0	15.0	−18.7
	120		120	68.8		
Isocit	120			60.2		
		120		1.9	39.7	
			120	1.0		
	120	120		44.2	7.1	−26.5
	120		120	61.3		
Isocit	120			69.0		
		120		1.3	35.2	
	120	120		50.3	7.2	−27.1
	60			33.9		
		60		1.5	31.1	
	60	60		26.1	9.9	−23.1
α-KG	120			63.0		
		120		0.9	37.7	
			120	1.2		
	120	120		32.6	10.9	−48.2
	120		120	45.5		
α-KG	120			65.7		
		120		1.1	26.2	
	120	120		45.1	13.1	−31.3
Succ	120			9.0		
		120		0.3	12.4	
			120	1.6		
	120	120		2.6	10.2	−71.1
	120		120	3.6		
	120			14.5		
		120		0.7	11.9	
	120	120		4.0	5.9	−72.4
	120		120	9.8		

[a] Incubation medium as described in Materials and Methods. Temperature was maintained at 37° for 30 min. Abbreviations: isocit, isocitrate; α-KG, α-ketoglutarate; succ, succinate. All are present as 10 mM. [b] Per cent change refers to the per cent change in corticosterone formed. Calculations based on the following equation: % change = [B from DOC] − [B from DOC plus progesterone]/B from DOC.

The effect of DOC on the 11β-hydroxylation of progesterone was always inhibitory regardless of the additive supplying the reducing equivalents (Tables I and II). Pregnenolone had neither an inhibitory influence when used with oxidizable substrates (Table I) nor a stimulatory action in the presence of TPNH and Ca^{2+}, suggesting that the progesterone effect is not one of merely increasing total steroid concentrations in the incubation medium. Pregnenolone was also ineffective in causing any significant variation in 11β-hydroxyprogesterone production (Tables I and II).

Time and concentration studies were carried out to examine what influence these parameters exerted on the paradoxical stimulation or inhibition of DOC to B as related to the nature of the additive supplying the reducing equivalents. Increasing DOC concentration with and without progesterone (60 μg) resulted in increased B production at all levels, but significantly less conversion was noted when the two steroids were incubated together in the presence of isocitrate (Figure 1). Conversely, TPNH- and Ca^{2+}-supported hydroxylations showed a higher conversion of DOC into B when progesterone was present (Figure 1). These relationships held true at all concentrations of steroids tested.

TABLE II: Steroid 11β-Hydroxylation by Rat Adrenal Mitochondria Incubated with Deoxycorticosterone, Progesterone, and Pregnenolone in the Presence of TPNH and Ca^{2+} or Supernatant from Various Species.[a]

Additions	Steroid Added (μg/tube)			Steroid Formed (μg/tube)		% Change[b]
	DOC	Proges-terone	Pregnen-olone	Corticos-terone	11β-OH Progesterone	
TPNH	120			11.6		
		120		2.0	18.4	
	120	120		19.9	13.2	+71.5
TPNH + Ca^{2+}	120			13.6		
		120		3.7	17.2	
	120	120		23.7	7.6	+74.2
		60		1.1	17.0	
	120	60		16.2	9.7	+19.1
TPNH + Ca^{2+}	120			10.8		
		120		1.0	15.6	
			120	3.0		
	120	120		16.7	11.5	+54.6
	120		120	11.4		
TPNH + Ca^{2+}	120			13.7		
		120		2.6	16.6	
		120	120	2.2	16.1	
	120	120		19.1	9.45	+39.4
TPNH + Ca^{2+}	60			17.3		
		120		1.7	22.2	
	60	120		25.6	17.1	+48.5
	60	60		23.9	18.5	+37.0
	120	120		23.0	16.2	+33.5
Sup (rat)	120			19.9		
		120		5.0	22.4	
			120	5.0		
	120	120		10.1	10.9	−49.2
	120		120	17.7		
Sup (rat)	120			16.3		
		120		3.0	31.4	
	120	120		8.9	21.0	−45.4
Sup (GP)	120			20.1		
	120	120		8.9	13.65	−55.7
Sup (GP)	120			20.1		
	120	120		16.7	11.8	−16.9
Sup (cow)	120			28.6		
		120		3.2	17.9	
			120	2.6		
	120	120		16.7	12.6	−41.6
	120		120	25.9		

[a] Incubation conditions as described in Materials and Methods. TPNH (800 μg) added where indicated; Ca^{2+} concentration is 5.5 μM. Abbreviations: Sup, supernatant from P_4 centrifugation (30,000 rpm for 1 hr); GP, guinea pig supernatant. [b] Per cent change as in Table I.

Incubations with all oxidizable substrates (isocitrate, succinate, α-ketoglutarate, and malate) and supernatants projected the same picture although not diagrammed here.

When increasing concentrations of progesterone were incubated with DOC there was a marked inhibition of 11β-hydroxyprogesterone formed with no distinction with regard to the source of reducing equivalents (oxidizable substrates, supernatant, or exogenous cofactor and Ca^{2+}; Figure 2). When DOC was added to the incubation contents, there was always a diminished 11β-hydroxylation of progesterone.

A closer examination of the progesterone effect shows that addition of increasing amounts of this

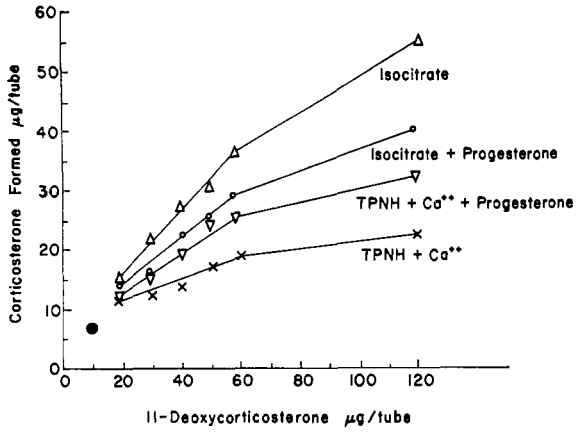

FIGURE 1: The effect of progesterone on the formation of corticosterone from various concentrations of DOC, as incubated with isocitrate or TPNH and Ca^{2+}.

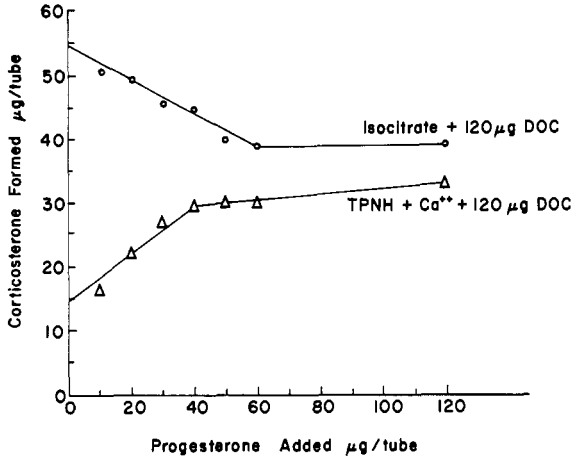

FIGURE 3: Corticosterone production as influenced by various concentrations of progesterone incubated with isocitrate or TPNH and Ca^{2+}.

steroid to a constant DOC concentration causes near-linear inhibition of the DOC to B reaction with oxidizable substrates (or high-speed supernatant) and a near-linear stimulation of the hydroxylation with exogenous TPNH and Ca^{2+} (Figure 3). This inhibition and stimulation reaches a maximum at about 50–60 μg of progesterone or approximate one-half equimolar amounts. The reverse situation, adding increasing DOC concentrations to a constant progesterone amount results in inhibition of 11β-hydroxyprogesterone production when up to 60 g of DOC was added, regardless of the source of reducing equivalents (Figure 4).

The relationship between time and 11β-hydroxylation of DOC and progesterone showed that corticosterone was produced both, more rapidly and more abundantly, than 11β-hydroxyprogesterone at all intervals tested (Figure 5). The conversion of DOC into B was near linear for the first 15 min without progesterone added and the first 20 min with progesterone. However, the conversion of DOC into B with progesterone was less over the full 30-min incubation period range. Since the pattern of steroid hydroxylations obtained was the same at each time interval over the 30-min incubation period range, that is, inhibition of DOC into B by progesterone and inhibition of progesterone into 11β-hydroxyprogesterone by DOC, the 30-min incubation period can still be used for comparison of the effects of these steroids on one another and is also consistent with the 30-min incubation period used to report data in a previous investigation (McCarthy and Péron, 1967). As expected, DOC markedly inhibited the production of 11β-hydroxyprogesterone

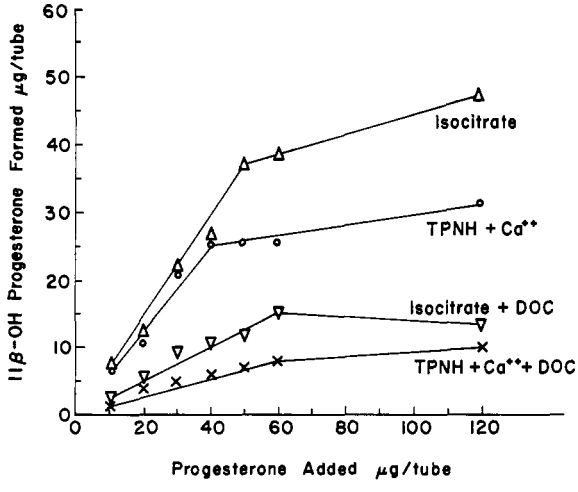

FIGURE 2: The effect of DOC on the formation of 11β-OH-progesterone from various concentrations of progesterone, as incubated with isocitrate or TPNH and Ca^{2+}.

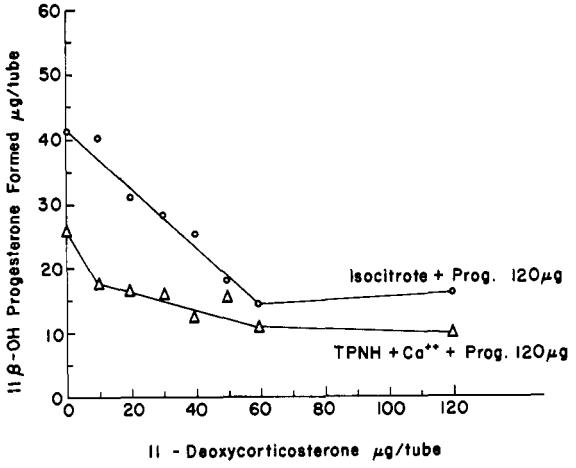

FIGURE 4: 11β-OH-Progesterone production as influenced by various concentrations of DOC incubated with isocitrate or TPNH and Ca^{2+}.

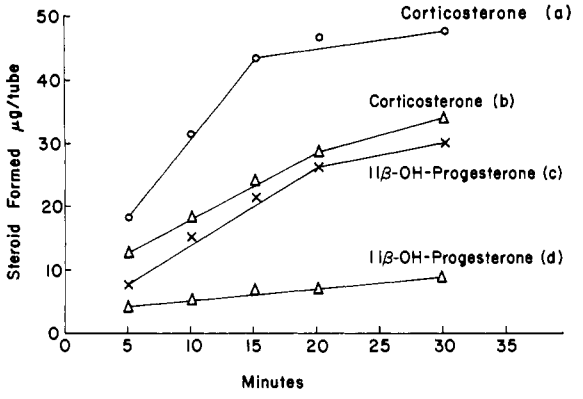

FIGURE 5: The effect of various steroid combinations on the 11β-OH-hydroxylation of DOC and/or progesterone incubated with isocitrate. (a) 120 μg of DOC/tube, corticosterone measured; (b) 120 μg of DOC and 120 μg of progesterone per tube, corticosterone measured; (c) 120 μg of progesterone/tube, 11β-hydroxyprogesterone measured; and (d) 120 μg of progesterone and 120 μg of DOC per tube, 11β-hydroxyprogesterone measured.

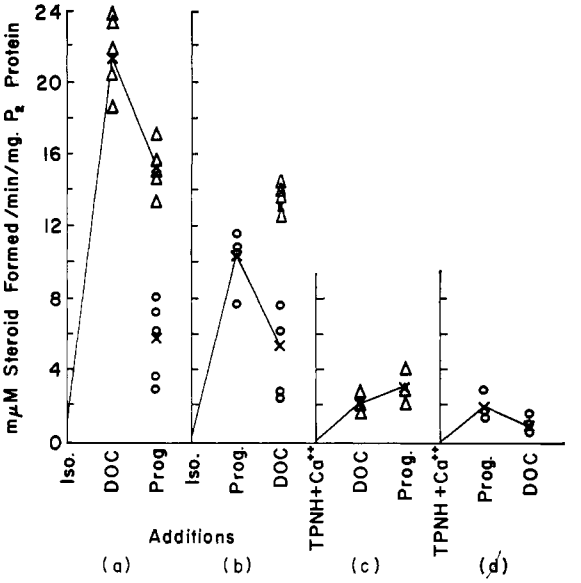

FIGURE 6: The effect of various steroid combinations on the 11β-hydroxylation of DOC and/or progesterone incubated with isocitrate or TPNH and Ca^{2+}. Rates of steroid hydroxylations were obtained by analyzing 0.20-ml aliquots of the incubation medium taken 5 min after each steroid addition. (Δ) Corticosterone; ($\circ$) 11β-OH-progesterone.

at each time period. All of these time studies were carried out with isocitrate as the additive in the presence of 0.68 mg of P_2 protein/tube. Increasing the quantity of mitochondrial protein present in each tube extended the curves to higher productions of steroid converted and at slightly faster rates, but the results obtained were essentially the same as those just discussed.

The rates of hydroxylation of DOC and progesterone are also presented in Figure 6. These data show (Figure 6a) a reduction of B production (from over 21 mμ-moles/min per mg of P-2 protein) of almost 30% by adding progesterone to DOC in an incubation using isocitrate as a reducing agent.

To be noted is that the sequence of addition of the steroid did not markedly alter the steroid conversion rate of each steroid when both were present in equimolar amounts (Figure 6a,b). Thus a similar inhibition of 11β-hydroxylation of progesterone is observed following the addition of DOC (from about 10 mμmoles/min per mg of P-2 protein to about 5 mμmoles/min per mg of P-2 protein). The TPNH and Ca^{2+} supported stimulation of DOC to B is readily apparent (Figure 6c). The conversion of DOC into B with exogenous cofactor and Ca^{2+} was increased from 2 to >3 mμmoles/min per mg of P-2 protein. The stimulation did not hold true for the 11β-hydroxylation of progesterone which was, as expected from the other incubation experiments (Tables I and II), decreased by the addition of DOC (Figure 6d).

As corollary to these results, the oxygen consumption of the mitochondria was also determined (Figures 7 and 8). Results obtained at both 25 and 37° showed that a reduction in oxygen consumption occurred following the addition of progesterone to DOC incubated with isocitrate (all oxidizable substrates and supernatant fractions acted in a similar manner). On the other hand, a slight stimulation of oxygen uptake was observed when DOC was added to progesterone incubated with isocitrate (Figure 7b). With exogenous TPNH and Ca^{2+} (Figure 8a), an increased oxygen utilization resulted upon addition of progesterone to DOC incubations. No significant effect was obtained when DOC was added to the oxygraph cells containing progesterone and TPNH and Ca^{2+} (Figure 8b).

The authors wish to point out that the figures illustrated herein are actual. They are representative of many duplicate experiments, and where isocitrate is listed the pattern of results obtained with all oxidizable substrates tested (α-ketoglutarate and succinate), and the supernatant fraction from all three species of animals (rat, guinea pig, and bovine) was similar.

Discussion

An analysis of the preceding results must attempt to reconcile the progesterone inhibition of the 11β-hydroxylation of DOC, when oxidizable substrates are the source of reducing equivalents, and the progesterone stimulation of the conversion of DOC into corticosterone in the presence of exogenous reduced cofactor and low calcium concentrations (Tables I and II). Even though the absolute values of corticosterone production are considerably higher when isocitrate and α-ketoglutarate are the additives, than with succinate or TPNH and Ca^{2+}, the demonstration of the dual

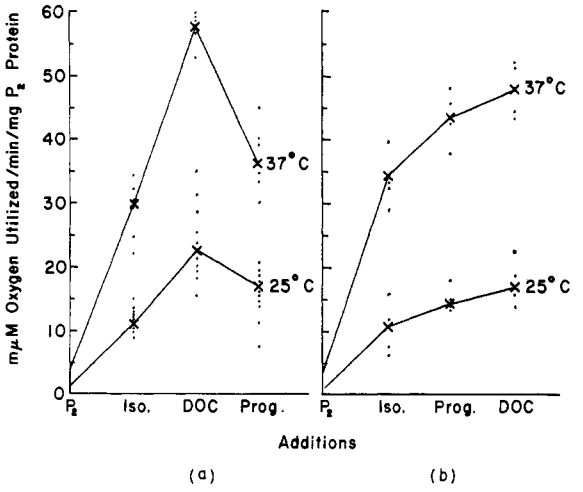

FIGURE 7: The effect of various steroid combinations on oxygen utilization by mitochondria incubated with isoctitrate.

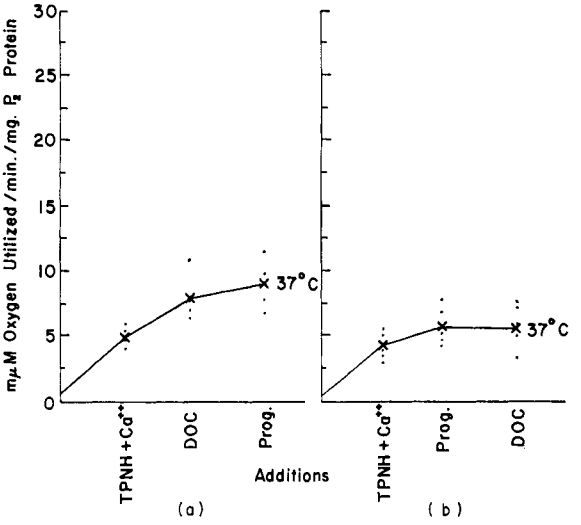

FIGURE 8: The effect of various steroid combinations on oxygen utilization by mitochondria incubated with TPNH and Ca^{2+}.

effect of progesterone on 11β-hydroxylation of DOC is obvious. This is clearly seen when a comparison of the per cent change in B formed is made at any production level. This indicates that it is not likely that the degree of reducing pressure (intramitochondrial TPNH) maintained by the various additives comes under the direct influence of progesterone.

A possible explanation of this phenomenon is the suggestion that progesterone may influence the rate of DOC uptake. However, the entry of DOC into the mitochondria is almost instantaneous (Purvis *et al.*, 1968) and thus reduces the possibility of progesterone increasing the rate of DOC transport which would account for the positive per cent change in B production with reduced exogenous cofactor. Conversely, it may be argued that progesterone decreases the rate of DOC uptake in the presence of the oxidizable substrates like isocitrate and succinate and accounts for the progesterone inhibition of the DOC to B reaction. However, the addition of DOC to the incubation media was always in excess amounts making it unlikely that the concentration of DOC would be limiting. Also, the near-linear inhibition of B formation observed when either fixed amounts of progesterone were incubated with varying DOC concentrations, or fixed DOC concentrations incubated with increasing progesterone doses in the presence of isocitrate, argues against this point (Figures 1 and 3). It also appears unlikely that changes in the structure of the mitochondria brought about by large amplitude swelling, as a result of 5 μM Ca^{2+} concentrations, would account for the aforementioned effects of progesterone on DOC transformation into B, when exogenous TPNH was the source of reducing pressure. Little or no swelling has been observed in rat adrenal gland mitochondria at this concentration of Ca^{2+} (Péron *et al.*, 1965a,b). It should be recalled also, that when Ca^{2+} levels were increased substantially beyond 100 μM (McCarthy and Péron, 1967), there resulted a significant inhibition of the DOC to B reaction when the incubations were carried out in the presence of progesterone.

On the other hand, if exogenous TPNH at this low Ca^{2+} concentration enters the mitochondria at locations which are different from endogenously produced reduced cofactor (provided by added oxidizable substrates), then the effect of progesterone stimulation might be one of influencing the entrance of the exogenous cofactor to these sites as a result of a slight mitochondrial swelling which was observed when DOC was incubated in the presence of progesterone (McCarthy and Péron, 1967). The lack of any effects by pregnenolone, the immediate precursor of progesterone, on 11β-hydroxylation of DOC under all conditions of incubation, indicates that the progesterone effect is quite specific.

Still another possibility for the observed progesterone effect is that this steroid inhibited TPN-linked isocitrate dehydrogenase or the respective dehydrogenases involved in α-ketoglutarate or succinate oxidation and thus explain a lowered DOC conversion into B with isocitrate and the other oxidizable substrates. This would also account for the lack of an inhibitory effect observed with exogenous TPNH.

McCarthy and Péron (1967) mentioned the possibility of competitive inhibition as an explanation for the consistantly lowered 11β-hydroxylation of progesterone after DOC additions. Attempts to plot results (Lineweaver–Burke plots) at 2.5 and 10 min with all of the various steroid combinations, showed the inhibition to be of the mixed type, and not a clear competitive inhibition. This mixed-type inhibition was even more evident for the inhibition of DOC to B with oxidizable substrates.

Oxygen-uptake studies under the various incubation

conditions provided some information related to the utilization of the different additions with respect to oxygen consumption for steroid hydroxylations. In the presence of isocitrate, the mean value obtained for the rate of B and 11β-hydroxyprogesterone production (Figure 6a,b) on a millimicromolar basis, accounts for the increased O_2 uptake above that found with isocitrate alone after addition of the two steroids (Figures 7a,b). The net O_2-uptake rate noted after DOC addition (Figure 7a) is somewhat higher on a millimicromolar basis than the B production (Figure 6a). This is not surprising because as previously reported (McCarthy and Péron, 1967), 18 OH-DOC is also produced as a result of 18-hydroxylation of DOC and this reaction requires molecular O_2. On the other hand, the rate of O_2 uptake in the case of 11β-hydroxylation of progesterone is more stoichiometric with 11β-hydroxyprogesterone production (Figures 6b and 7b; about 10 mμmoles of O_2 and 10 mμmoles of 11β-hydroxyprogesterone per min per mg of P_2 protein, respectively). This is due to the fact that little or no 18-hydroxylation of progesterone occurred (unpublished observation). The inhibitory effect on 11β-hydroxylation of DOC which additions of progesterone brought about was also reflected in a decreased rate of O_2 uptake. The decreased O_2 uptake and rate of 11β-hydroxylation of DOC probably indicate the degree of electron-flow inhibition along the P_{450} chain. That progesterone does not bring about complete inhibition of the DOC into B conversion was also indicated (Figure 7b) by the increased O_2-uptake rate when DOC was added after progesterone.

The oxygen-uptake studies in the TPNH and Ca^{2+} system (Figure 8a,b) also supported our steroid analysis studies of Tables I and II since addition of progesterone after DOC or addition of DOC after progesterone did not lead to a decrease in O_2 uptake. It will be recalled that in the presence of TPNH and Ca^{2+} progesterone increased the amount of DOC converted into B (Table II and Figure 6c). On the other hand, the action of DOC on the 11β-hydroxylation of progesterone was consistently inhibitory without regard to whether isocitrate was the oxidizable substrate providing intramitochondrial TPNH or whether exogenous TPNH and Ca^{2+} was added to the system (Figure 6d).

These data do not permit us, at the moment, to resolve the enigma of the progesterone inhibition on DOC into B transformation when oxidizable substrates like isocitrate provide intramitochondrial TPNH (reducing pressure) nor the increased stimulation of this reaction when reducing pressure is provided in the form of exogenously added TPNH. The inhibition of O_2 uptake when progesterone is used in the first aforementioned instance and as mentioned before, probably indicates that the steroid inhibits electron flow along the P_{450} chain which is necessary for 11β-hydroxylation of DOC (Omura *et al.*, 1965, 1966; Harding *et al.*, 1965). On the other hand, since balanced O_2-uptake studies were not carried out to assess the function or participation of the classical electron chain in these steroid hydroxylations, an inhibitory effect of progesterone on the respiratory chain cannot be ruled out. To determine the stoichiometry between steroid interconversion, O_2 utilization and the extent of participation of the P_{450} and respiratory chain, it will be necessary to ensure that little O_2 is taken up by the respiratory chain and that all steroid products of DOC and progesterone are measured (Purvis *et al.*, 1968).

The results of studies carried out with the high-speed supernatant fraction are indicative that reducing equivalents provided by this fraction come about as a result of oxidation of substrate(s) present in this fraction. Because progesterone inhibits the mitochondria supernatant fraction combination in the DOC to B reaction, in a manner similar to when isocitrate is added to the mitochondria, it is tempting to believe that these substrate(s) may be Krebs cycle intermediates. More direct evidence for this belief is presently being sought in studies being carried out in this laboratory. The above facts also indicate that hydroxylation of DOC to B when supernatant fraction is added is not brought about by the TPNH which might be present in it at the time of its addition (Péron and McCarthy, 1968). If this had been so, an activation rather than an inhibition of the DOC to B reaction could be expected when progesterone was added to mitochondria incubated in the presence of DOC and supernatant fraction.

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Mechanisms of Steroid Oxidation by Microorganisms. XIII. C_{22} Acid Intermediates in the Degradation of the Cholesterol Side Chain*

Charles J. Sih, K. C. Wang,† and H. H. Tai

ABSTRACT: Experimental evidence is herein presented to show that C_{22} acids are key intermediates in the microbiological degradation of the cholesterol side chain. Exposure of 19-hydroxycholest-4-en-3-one to microorganisms of the genus *Nocardia* produced four new C_{22} acids besides estrone; the chemical structures of these metabolites have been characterized as 3-hydroxy-19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oic acid (IIa), 3-hydroxy-19-norbisnorchola-1,3,5-(10)-trien-22-oic acid (IIIa), 3-hydroxy-19-norbisnorchola-1,3,5(10),9(11)-tetraen-22-oic acid (IV), and 3-oxo-19-hydroxybisnorchol-4-en-22-oic acid (Va). From radioactive tracer experiments, it was established that the three-carbon side chain of C_{22} acid can be cleaved under anaerobic conditions to yield propionic acid and 17-keto steroids.

Although the side chain of cholesterol is selectively oxidized, in a stepwise manner, leading to the formation of bile acids and the various classes of steroid hormones, it is believed that relatively little breakdown of the steroid nucelus occurs in mammalian tissues (Talalay, 1957). In contrast, a variety of microorganisms are capable of utilizing sterols, bile acids, and steroid hormones for growth (Turfitt, 1944; Halperin *et al.*, 1954; Schatz *et al.*, 1949). Under suitable conditions, certain microorganisms have the capacity to degrade sterols, such as cholesterol, completely to carbon dioxide and water (Turfitt, 1947). Although there has been considerable progress in elucidating the pathway for the breakdown of the steroid skeleton in recent years (Dodson and Muir, 1961; Sih *et al.*, 1966; Gibson *et al.*, 1966; Lee and Sih, 1967), the mechanism of microbial degradation of the cholesterol side chain is not well understood until now. This paper deals with the identification of C_{22} acids as key intermediates in the breakdown of the cholesterol side chain by microorganisms and the mode of their conversion into 17-keto steroids. A preliminary communication on this subject has already appeared (Sih *et al.*, 1967).

Previous publications have shown that 19-oxygenated derivatives of cholesterol could be efficiently transformed by microorganisms into 17-keto steroids (Sih *et al.*, 1967). By shortening the incubation period to 48 hr, 19-hydroxycholest-4-en-3-one (I) was converted by *Nocardia restrictus* (ATCC 14887) into four acidic products (IIa, IIIa, IV, and Va) (Chart I) besides estrone.

3-Hydroxy-19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oic Acid (IIa). The first product (mp 241–243°) was initially assigned the structure 3-hydroxy-19-norbisnorchola-1,3,5(10),17(20)-tetraen-22-oic acid (IIa) on the basis of the following data. Molecular weight determination by mass spectrometry showed 326 for the parent compound and 340 for its methyl ester derivative; its ultraviolet absorption peaks ($\lambda_{max}^{CH_3OH}$ 225 mμ (ϵ 19,500) and 280 mμ (ϵ 2300)) are characteristic for a phenol and an α,β,β-trisubstituted α,β-unsaturated carboxylic acid chromophore; its infrared spectrum showed bands at λ_{max}^{Nujol} 2.98, 5.99, 6.21, 6.30, and 6.67 μ; its nuclear magnetic resonance spectrum showed bands at τ 9.21 (3 H, singlet, CH_3 at C-18), 8.11 (3 H, singlet, CH_3 at C-21), 3.57 (singlet), 3.48 (doublet, J = 9 cycles/sec), and 2.97 (doublet, J = 9 cycles/sec) (3 H, aromatic protons).

An analysis of mass spectrum of IIb supports the assigned structure. The spectrum (Figure 1) showed a parent ion peak (*m/e* 340) with peaks at *m/e* 325, 293, and 265, corresponding to the successive loss of CH_3, $HOCH_3$, and CO. The peaks at *m/e* 309 and 281 represent the loss of OCH_3 and $COOCH_3$, respectively, from the parent ion. The *m/e* 253 peak, corre-

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† Present address: Institute of Steroid Chemistry, Syntex Research, Palo Alto, Calif.