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STEROID HYDROXYLATIONS BY GUINEA-PIG ADRENAL TISSUE FRACTIONS

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SUMMARY

The effect of oxidizable substrates, α-ketoglutarate and isocitrate on 11β-hydroxylation in guinea-pig adrenal mitochondria has been studied. These substrates supported the conversion of Compound S (17α,21-dihydroxy-pregnene-3,20-dione) into cortisol as well as exogenously added NADPH in Ca^{2+}-swollen mitochondria. Progesterone was also hydroxylated at the C-11β position but not at the C-21 position. Microsomes, on the other hand, when NADPH was added, converted pregnenolone, progesterone and 17α-hydroxyprogesterone into Compound S, but showed no steroid 11β-hydroxylating activity. Evidence obtained in incubations carried out with α-ketoglutarate and isocitrate in the presence of 2,4-dinitrophenol, oligomycin, amytal and antimycin A indicate that α-ketoglutarate utilization for steroid 11β-hydroxylation is dependent on activity of the classical electron chain. This activity can be related to high energy intermediates possibly needed for NADPH reduction arising from NADH oxidation *via* the energy-requiring transhydrogenase reaction. These reactions do not appear necessary for isocitrate utilization and isocitrate oxidation probably gives rise to intramitochondrial NADPH reduction as a result of a $NADP^+$-linked isocitrate dehydrogenase. Data obtained in oxygen uptake studies with an antimycin A blocked system supplied with α-ketoglutarate, are in accordance with this conclusion. The high-speed supernatant fraction (103000 × *g*) could partially replace α-ketoglutarate, isocitrate or Ca^{2+} + NADPH, indicating that it contains a factor(s) (the physiological substrate?) which brings about intramitochondrial NADPH.

INTRODUCTION

In recent years, there have been several publications concerning utilization of reduced pyridine nucleotides in steroid hydroxylations by adrenal tissue of several species of animal. The picture which has emerged, is that NADPH is involved in several of the steroid hydroxylations occurring at the mitochondrial and microsomal levels by a process requiring electron flow along a chain whose components are a flavoprotein having NADPH-diphorase activity, a brown-colored non-heme iron protein and a cytochrome having a maximum 450 mμ absorption peak in the reduced state (P-450)[1-10]. By virtue of this process in the presence of molecular O_2, the terminal oxygen activator P-450 and the mixed function oxidases (steroid 11β-hydroxylase,

21-hydroxylase, 18-hydroxylase), introduction of the hydroxyl group at certain positions of the steroid nucleus occurs. Although further clarification of the exact mechanism is needed, whereby electron flow proceeds in the above as along the components of the classical respiratory chain, there is little evidence available at present which indicates that NADPH is not involved in adrenal mitochondrial steroid hydroxylations in the rat and the ox. Whereas oxidation of succinate and NAD^+-linked substrates appear to stimulate 11β-hydroxylation of deoxycorticosterone in bovine adrenal mitochondria by the aforementioned mechanism subsequent to NADPH formation (*via* transhydrogenation)[3,11] in the rat, isocitrate appears to be the substrate which is most readily utilized for 11β-hydroxylation of deoxycorticosterone[12–14]. Indeed, in the rat adrenal, it has been shown that mitochondrial isocitrate dehydrogenase is mostly $NADP^+$-linked[13]. This leads to intramitochondrial NADPH production which is then utilized by the P-450 chain for corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione) production from deoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione). Further evidence implicating NADPH in steroid 11β-hydroxylations was also obtained in Ca^{2+}-swollen rat adrenal mitochondria[12,14–17].

The present investigation, using the guinea pig as the source of adrenal tissue, was initiated following a preliminary report by SPATZ AND HOFMANN[18], and their recent publication[19] which indicated that adrenal steroid C-11β-hydroxylation may not be mediated by NADPH in this species of animal. Our findings obtained with Ca^{2+}-swollen guinea-pig mitochondria do not support such a conslusion and in contradistinction indicate that NADPH is involved in steroid 11β-hydroxylation of progesterone (4-pregnene-3,20-dione), deoxycorticosterone and Compound S (17α,21-dihydroxy-4-pregnene-3,20-dione) at the mitochondrial level as well as being involved in 21-hydroxylation of progesterone at the microsomal level. In this study, we also present an application of the fluorometric method of SILBER, BUSCH AND OSLAPAS[20] which can be used for the determination of small amounts of cortisol in samples of biological material containing relatively large amounts of Compound S.

EXPERIMENTAL PROCEDURE

All guinea pigs used were males weighing 350–450 g and were sacrificed by a blow administered to the head. The adrenal glands were excised immediately, stored in ice-cold 0.25 M sucrose and cleaned of adhering fat. The adrenal glands were homogenized in exactly the same manner as previously reported for preparing rat adrenal homogenates[16] in a mixture of Tris buffer (pH 7.4), nicotinamide and sucrose (no EDTA added, see ref. 16). The final concentration of these substances in the homogenate was 15, 20 and 250 mM, respectively, and there was approximately 450 mg wet weight of adrenal tissue per 1.00 ml of homogenizing medium. Homogenization was carried out by subjecting the tissue to 10–20 passes in an all-glass homogenizer with a motor-driven pestle. The homogenate was next centrifuged at 900 $\times$ *g* for 15 min and the resultant pellet (Pellet 1, P_1) discarded. The supernatant fraction was next centrifuged at 5000 $\times$ *g* for 15 min. The mitochondria[16] (Pellet 2, P_2) were washed twice with buffer–sucrose–nicotinamide mixture (mixture B) described above and recentrifuged at 5000 $\times$ *g*. After centrifugation of the supernatant fraction from the first 5000 $\times$ *g* centrifugation at 14500 $\times$ *g* for 15 min to eliminate "light" mitochondria and "heavy" microsomes, the resultant supernatant fraction was centri-

fuged for 60 min at 30000 × *g* to obtain the microsomes (Pellet 4, P_4). The latter had the same appearance as those obtained in the rat experiments and were clear reddish-brown in color and gel-like in consistency[16]. The supernatant fraction derived from this last centrifugation was utilized in some experiments and is designated as the high-speed supernatant fraction in the text, or abbreviated as Sup. in the Tables.

The experiments on swelling and steroid conversion were carried out simultaneously in a total volume of 1 ml in glass stoppered tubes fitting into the Coleman Junior spectrophotometer as previously reported[16]. When inhibitors were used (cyanide, oligomycin, *etc.*), these were always added to the tubes 7 min before the addition of oxidizable substrates (α-ketoglutarate, isocitrate) or the swelling inducing agent (calcium) as previously reported[12]. The final incubation medium contained 20 mM Tris (pH 7.4), 15.4 mM NaCl, 50.0 mM nicotinamide, 15.4 mM KCl, 10 mM Mg^{2+}, 0.10 % bovine serum albumin, 100 mM sucrose and other additions indicated in the tables. Proteins were determined by the method of LOWRY *et al.*[21], and each tube contained approximately 0.75 mg mitochondrial or 0.50 mg microsomal protein. Steroids (120 μg) were added to the incubation medium in 0.01 ml of an equal mixture propylene glycol and ethanol.

The amount of cortisol formed in the mitochondrial incubations from added Compound S was determined by fluorometry according to the procedure of SILBER, BUSCH AND OSLAPAS[20], with the following modification. Because the aqueous -H_2SO_4 reagent added in the last stage of the fluorometric procedure induces some fluorescence of Compound S, a carbon tetrachloride extraction (4.0 ml) was carried out after aliquoting the incubation media to the 13 % aqueous ethanol of the first stage of the fluorometric procedure[20,22]. This initial extraction removed most of the Compound S which remained unconverted to cortisol at the end of the incubation period. As can be seen in the example given in Table I, the respective fluorescence values for Compound S and cortisol are vastly different at the levels of concentrations shown, when the sensitivity knob of the photomultiplier was set at 25 and the meter multiplier switch set at 1.0 (Aminco-Bowman spectrophotofluorometer).

When the CCl_4 extraction step was omitted, the readings attributed to Compound S alone were about 5 times those reported in Table I. On the other hand, the readings obtained with cortisol only (in the range 0.2–3.2 μg) were not affected in any way. Thus, the extraction of the 13 % ethanolic phase with CCl_4 in the present study, removed the greater proportion of the Compound S which would interfere with the accurate fluorometric determination of cortisol, especially when the latter was present at low concentrations (0.2 μg). In any event, a Compound S standard

TABLE I

FLUORESCENCE OF CORTISOL AND REICHSTEIN'S COMPOUND S AFTER AN INITIAL CCl_4 EXTRACTION

Cortisol (F), μg	0.2	0.4	0.8	1.6
Fluorescence reading	9	19	39	74
Compound S (S), μg	3.0	6.0	12	24
Fluorescence reading	1.5	3	6	13
F + S reading*	9	20	46	91

* Reading obtained when F and S were present together and run through the procedure at the concentrations shown in the table.

curve was always carried out with amounts of this steroid which would be present in the aliquots taken of final incubation medium for the cortisol determination, assuming that there was no Compound S conversion into cortisol. Thus, if 0.02, 0.05 and 0.10 ml of the incubation medium after the final 30-min incubation was used for the analysis of cortisol, 2.4, 6.0 and 12.0 μg of Compound S were carried through the fluorometric procedure and the readings obtained with the respective samples subtracted from the values obtained with the aliquots of the incubation medium. It is obvious that the values obtained with this method are not absolute, especially in those experiments wherein there was a substantial amount of Compound S conversion into cortisol. However, the relatively small readings (3 and 6, see Table I) obtained with the Compound S (*i.e.* when 0.05 and 0.10 ml are used for the cortisol determination) *vs.* the high readings (58 and 116) obtained in these samples when cortisol was produced at a high level (*e.g.* 25 μg/ml final incubation medium) only introduces a slight error (underestimation) in the determination of the cortisol by this method. In order to confirm these deductions, experiments were conducted on random samples (see Table II) wherein the cortisol formed was isolated on paper chromatograms after running in the conventional Bush B_5 system. For these chromatographic runs the residual incubation medium, after taking the aliquots for the direct fluorometric determination of cortisol, was extracted with 8 ml (2 $\times$ 4 ml) of CH_2Cl_2 after addition of 0.10 μg of [$^{14}C_4$]cortisol (11β,17α,21-trihydroxy-4-pregnene-3,20-dione) specific activity 42.5 μC/mmole. The radioactive tracer amounts of cortisol served as a guide to determine recovery values. The respective CH_2Cl_2 extracts were pooled and brought to dryness under N_2 at 37° and applied to paper chromatograms and run in the Bush B_5 system. Elution of the cortisol spots, after careful visual examination of the chromatograms under ultraviolet light, was next carried out with methanol. The final methanol eluates were finally adjusted to 10.0 ml exactly with methanol and separate aliquots (0.10, 0.20 ml) taken for counting in a Packard Tri-Carb liquid scintillation spectrometer model 314-DC, after addition of 10.0 ml of the usual counting fluid. Samples of the standard [4-^{14}C]cortisol (0.10 μg) were also made up to 10.0 ml in methanol without being subjected to extraction or paper chromatography. Per cent recovery values of the eluted cortisol spots from the paper chromatograms were obtained by comparing the counts obtained in the methanol aliquots derived from the paper chromatographic elutions and those in equal methanol aliquots of the standard just described. In the eleven samples thus analyzed the mean recovery value was 59 %.

The method for determining 11β-hydroxyprogesterone (11β-hydroxy-4-pregnene-3,20-dione) was that outlined previously by McCARTHY AND PÉRON[17].

Changes in level of nicotinamide–adenine dinucleotide reduction (steady states) in guinea-pig mitochondria incubated in the presence of succinate and isocitrate were followed in an Eppendorf fluorometer equipped with zero suppression features and recorded continuously with the use of an attached Minneapolis-Honeywell recorder. An alteration in fluorescence equivalent to 0.05 mμM of nicotinamide–adenine dinucleotide in 2 ml of medium can be determined with the settings used.

Mitochondrial NAD^+, $NADP^+$, NADH and NADPH was estimated specifically before and after incubation with substrates (isocitrate and α-ketoglutarate) by the enzymatic method of COLOWICK AND KAPLAN[23] as described by PURVIS[24].

The rate of oxygen utilization by mitochondrial suspensions was measured

polarographically at 37° using the vibrating micro-platinum electrode of a Gilson Medical Electronics Model KM oxygraph.

RESULTS

The effect of adding various amounts of NADPH on 11β-hydroxylation of Compound S is shown in Table II. Without the addition of Ca^{2+} little 11β-hydroxylation of Compound S occurred. The response of the system appears to be dependent on the concentration of NADPH even at the levels of Ca^{2+} (2.2 mM) which are usually present in Krebs–Ringer bicarbonate buffer. An effect of Ca^{2+} on mitochondrial swelling is clearly seen (Fig. 1) whereas little or no swelling of the guinea-pig mitochondria occurred in the presence of NADPH alone. The oxidation of succinate and malate which are both known to lead to the production of reducing equivalents in rat adrenal mitochondria and are utilized for 11β-hydroxylation of deoxycorticosterone[12,13], brought about little 11β-hydroxylation of Compound S in these experiments (Table II). On the other hand, isocitrate and α-ketoglutarate were utilized much more efficiently and considerable 11β-hydroxylation of Compound S took place.

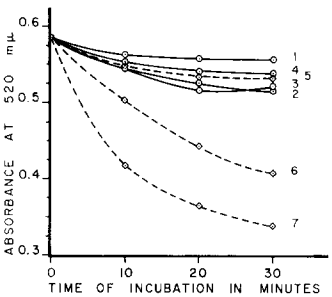

Fig. 1. Course of swelling of guinea-pig adrenal mitochondria brought about by Ca^{2+}. Curve 1, incubation of P_2 alone; Curves 2, 3 and 4, in the presence of 100, 400 and 800 μg NADPH, respectively; Curves 5, 6 and 7, in the presence of 800 μg NADPH and 330 μM, 2.2 mM and 11 mM Ca^{2+}, respectively. Identical Ca^{2+}-swelling curves were obtained in the presence or absence of NADPH.

It is to be noted that addition of ATP had little effect on isocitrate and α-ketoglutarate utilization, but did affect succinate and malate utilization. Substances which block the respiratory chain had little effect on isocitrate utilization whereas antimycin A decreased the amount of cortisol to 44 % of the control level when α-ketoglutarate was used. Ferricyanide had little effect on isocitrate- and α-ketoglutarate-supported 11β-hydroxylation of Compound S, whereas a pronounced inhibitory effect was obtained with the uncoupler 2,4-dinitrophenol when α-ketoglutarate was the substrate. 2,4-Dinitrophenol had a much smaller effect on isocitrate utilization whereas oligomycin had a slight inhibiting effect with both Krebs-cycle intermediates.

As previously mentioned in EXPERIMENTAL PROCEDURE, analysis of the cortisol following its chromatographic separation on paper chromatograms was carried out. The values obtained for the cortisol eluates (Table II) are shown in parentheses and are in good agreement with those obtained by the direct fluorometric assay of cortisol carried out on aliquots of the incubation medium. A careful examination of these

TABLE II

THE CONVERSION OF COMPOUND S INTO CORTISOL BY GUINEA-PIG ADRENAL MITOCHONDRIA INCUBATED IN THE PRESENCE OF CALCIUM + NADPH AND KREBS-CYCLE INTERMEDIATES

The final incubation was carried out as outlined in EXPERIMENTAL PROCEDURE at 37°. KCN was brought to pH 8.1 just before its addition. Values in parentheses are those obtained by analyzing the cortisol eluted from paper chromatograms (see EXPERIMENTAL PROCEDURE).

Substrate or cofactor added	*μg cortisol per mg mitochondrial protein*						
	NADPH (100 μg)	*NADPH (400 μg)*	*NADPH (800 μg)*	*0.10 M succinate*	*0.10 M malate*	*0.10 M isocitrate*	*0.10 M α-ketoglutarate*
	0	—	—	0 (1.8)	0	0	0 (1.4)
120 μg Compound S	0	0.8	0.8	0.7	3.1	24.6 (20.4)	25.5 (19.9)
120 μg Compound S + Ca^{2+}, 220 μM	0.8	2.6	2.9 (2.1)	—	—	—	—
120 μg Compound S + Ca^{2+}, 2.2 mM	2.0	15.2	16.1 (14.4)	—	—	—	—
120 μg Compound S + Ca^{2+}, 11.0 mM	7.6	25.3	28.2 (23.5)	—	—	—	—
120 μg Compound S + ATP, 1 mM	—	—	20.2*	7.7 (7.7)	13.7 (14.3)	25.0	25.8 (26.1)
120 μg Compound S + antimycin A, 1 μg	—	—	—	0.7	2.1	22.3	11.3
120 μg Compound S + KCN, 3 mM	—	—	22.4*	0.4	1.0	24.1	24.2
120 μg Compound S + $K_3Fe(CN)_6$, 1 mM	—	—	1.8*	0.6	1.5	25.2	24.9
120 μg Compound S + 2,4-dinitrophenol, 500 μM	—	—	23.0*	0.4	1.1	17.9	3.5
120 μg Compound S + oligomycin, 2 μg	—	—	—	0.6	2.8	23.0	19.3

* Separate experiment.

chromatograms under ultraviolet light and then spraying with the alkaline blue tetrazolium reagent[25] failed to reveal any substances (ultraviolet light absorbing, blue tetrazolium-reducing or soda fluorescing) other than cortisol and unconverted Compound S.

To test the potential of the 11β-hydroxylase activity of the guinea-pig adrenal mitochondria, they were incubated in the presence of progesterone and deoxycorticosterone, respectively. In addition, the capacity of the supernatant fraction to replace NADPH + Ca^{2+}, α-ketoglutarate and isocitrate, was also investigated (Table III). In all cases, progesterone was efficiently converted to 11β-hydroxyprogesterone (11β-hydroxy-4-pregnene-3,20-dione, section II) whereas deoxycorticosterone was converted to the corresponding 11β-hydroxylated derivative, corticosterone (section III). The capacity of the supernatant fraction to provide reducing equivalents needed to drive 11β-hydroxylation of Compound S is also demonstrated (column I) as well as the effects brought about by addition of inhibitors (KCN, antimycin A), the uncoupler 2,4-dinitrophenol and the terminal electron acceptor $K_3Fe(CN)_6$. The identity of the analyzed cortisol (section I) and corticosterone (section III) was ascertained by paper chromatography. In all cases, therefore, the addition of the supernatant fraction to P_2 led to the formation of cortisol from Compound S. Incubation of P_2 with deoxycorticosterone under the conditions of the present experiment led to the formation of corticosterone only. No trace of 18-hydroxydeoxycorticosterone (18,21-dihydroxy-4-pregnene-3,20-dione) was seen on the paper chromatograms upon visual examination under ultraviolet light nor did subsequent spraying of the paper chromatograms with alkaline blue tetrazolium[26] reveal a fluorescing spot corresponding in mobility to 18-hydroxydeoxycorticosterone.

TABLE III

THE CONVERSION OF COMPOUND S INTO CORTISOL BY GUINEA-PIG ADRENAL MITOCHONDRIA INCUBATED WITH SUPERNATANT FRACTION. 11β-HYDROXYLATION OF PROGESTERONE AND DIOXYCORTICOSTERONE BY GUINEA-PIG ADRENAL MITOCHONDRIA INCUBATED IN THE PRESENCE OF NADPH + Ca^{2+} AND KREBS-CYCLE INTERMEDIATES

Conditions of incubation were those of Table II. Where added, NADPH = 800 μg and Ca^{2+} concentration = 11 mM. (1) Values obtained with the blue tetrazolium method[27]; (2) using the fluorescent method outlined in this paper (with CCl_4 extraction); (2a) same as 2 but no initial CCl_4 extraction; (3) radioisotope tracer method for 11β-hydroxyprogesterone[17]. Steroid products formed in columns I, II and III were cortisol, 11β-hydroxyprogesterone (designated under 3 of II) and corticosterone, respectively.

Additions	*μg steroid 11β-hydroxylated per mg mitochondrial protein*					*Additions*
	(I) P_2 + *Sup.** + *Compound S*	*(II)* P_2 + *progesterone*			*(III)* P_2 + *deoxycorticosterone*	
	2	*1*	*2*	*3*	*2a*	
—	16.4					
ATP, 1 mM	18.3	—	—	—	0.56	11 mM Ca^{2+}
2,4-Dinitrophenol, 500 μM	1.4	2.7	0.33	20.1	17.9	NADPH + 11 mM Ca^{2+}
KCN, 3 mM	9.2	2.3	0.0	28.2	21.4	Isocitrate, 10 mM
$K_3Fe(CN)_6$, 1 mM	14.9	2.3	0.0	23.1	22.0	α-Ketoglutarate, 10 mM
Antimycin A	9.5					

* An equivalent of 2.63 mg supernatant fraction protein was added in these incubations. No NADPH or Ca^{2+} added in this experiment.

TABLE IV

THE CONVERSION OF PREGNENOLONE, PROGESTERONE AND 17α-HYDROXYPROGESTERONE INTO COMPOUND S BY GUINEA-PIG ADRENAL MICROSOMES INCUBATED IN THE PRESENCE OF NADPH KREBS-CYCLE INTERMEDIATES AND SUPERNATANT FRACTION

In this table, 1, 2 and 3 = Expts. 1, 2 and 3, respectively. Each tube in the incubation contained the equivalent of 0.49 mg (Expt. 1), 1.59 mg (Expt. 2) and 0.45 mg (Expt. 3) microsomal (P_4) protein. In combination experiments (P_4 + Sup; microsomes + supernatant fraction) in addition to microsomes, each tube contained Sup. equivalent to 55.8 mg (Expt. 1), 78.6 mg (Expt. 2) and 14.8 mg (Expt. 3) of original wet weight of adrenal tissue. In columns I–IV the steroid formed was Compound S except in the case of P_2 + P_4 + Sup. + NADPH, bottom line (see text). The values shown in parentheses represent those obtained with the fluorometric procedure outlined in EXPERIMENTAL PROCEDURE. Other values are those obtained with the blue tetrazolium reagent[27]. Incubation conditions were those of Table II.

Additions	*μg hydroxylated steroid formed per incubation tube*											
	(I) None		*(II) Pregnenolene*			*(III) Progesterone*			*(IV) 17α-Hydroxy-progesterone*	*(V) Compound S*	*(VI) Deoxy-corticosterone*
	1	*2*	*1*	*2*	*3*	*1*	*2*	*3*	*3*	*1*	*1*
P_4 + NADPH, 800 μg	0.5 (0.3)	0.6	17.3 (0.7)	45.5	24.0 (1.5)	20.1 (0.6)	71.8	22.1 (6.5)	30.5 (0.7)	(2.0)	(1.2)
P_4 + NADPH, 800 μg + 11 mM Ca^{2+}	0.8 (0.3)	1.3	14.6 (0.7)	41.1	—	16.2 (0.5)	62.2	—	—	(1.6)	(1.2)
P_4 + α-ketoglutarate	—	—	—	3.6	0(0)	—	4.0	1.9 (1.0)	0 (0)	—	—
P_4 + isocitrate	—	0.8	—	4.3	2.1 (1.0)	—	4.9	3.9 (1.0)	3.2 (0)	—	—
Sup. + α-ketoglutarate	—	1.0	—	5.3	—	—	10.1	—	—	—	—
Sup. + isocitrate	—	0.3	—	2.9	—	—	11.9	—	—	—	—
P_4 + Sup.	1.7 (0.4)	1.6	9.4 (0.6)	21.5	—	10.2 (0.5)	21.3	—	—	(1.2)	(0.9)
P_4 + Sup. + α-ketoglutarate	3.1 (0.4)	—	8.7 (0.5)	21.5	—	9.9 (0.5)	23.0	—	—	(1.3)	(0.9)
P_4 + Sup. + isocitrate	2.8 (0.4)	0.8	21.5 (0.4)	34.2	—	43.7 (0.9)	74.5	—	—	(2.0)	(1.5)
P_2 + P_4 + Sup. + 800 μg NADPH	—	—		—	14.5 (14.7)	—	—	22.2 (14.5)	—	—	—

The data presented in Table IV show that the fraction labelled microsomes in our centrifugation method was able, in the presence of NADPH to efficiently hydroxylate progesterone at the C-17α and C-21 positions, and to also bring about the conversion of pregnenolone into Compound S *via* progesterone and 17α-hydroxyprogesterone formation. Since the blue tetrazolium-reducing method used[26,27] for analysis would not differentiate between Compound S, corticosterone or cortisol had they been produced, and the fluorometric procedure outlined in EXPERIMENTAL PROCEDURE would serve to indicate that only cortisol had not been formed, representative extracts of aliquots of the incubation media from each experiment carried out were subjected to paper chromatography. Only when the combination $P_2 + P_4$ + Sup. was used (bottom line, Table IV) was there any evidence that 11β-hydroxylation of the steroids added or formed occurred. Thus, no corticosterone nor cortisol were formed except in the aforementioned case. The fact that neither Compound S nor deoxycorticosterone were 11β-hydroxylated into cortisol or corticosterone also supported the above finding that P_4 and supernatant fractions were uncontaminated with mitochondria (P_2) which contains the 11β-hydroxylase enzyme.

A point of similarity between this microsomal preparation and those obtained previously with the rat[15] is the lack of any Ca^{2+} effect when incubated in the presence of NADPH.

While the addition of α-ketoglutarate or isocitrate to the supernatant or microsomal fractions alone had little effect on the steroid substrate transformation, it can be seen that the combination supernatant fraction + microsomes, is effective in bringing about the formation of Compound S from either pregnenolone or progesterone. Only isocitrate enhanced the supernatant effect and increased the amount of Compound S produced to about 8-fold over the control values (P_4 + isocitrate).

Since the amount of cortisol and corticosterone converted from Compound S and deoxycorticosterone, respectively (Tables II and III) reflect the generation and utilization of reducing power provided by oxidation of isocitrate and α-ketoglutarate by the guinea-pig adrenal mitochondria, attempts were made to measure the genera-

TABLE V

EFFECT OF α-KETOGLUTARATE, ISOCITRATE AND COMPOUND S ON STATE OF PYRIDINE NUCLEOTIDES IN GUINEA-PIG ADRENAL MITOCHONDRIA

Guinea-pig adrenal mitochondria were incubated at 37° for 15 min in the presence and absence of 10 mM α-ketoglutarate or isocitrate, respectively, in the buffer used for the usual incubations with steroids. Where indicated 330 μM of Compound S was present. The reaction was stopped by acid or boiling Na_2CO_3 and the pyridine nucleotides determined by specific enzymic fluorometric methods as described by PURVIS[21]. Values are in mμmoles/mg mitochondrial protein.

Addition	*NAD*$^+$	*NADH*	*NAD*$^+$ + *NADH*	*NADP*$^+$	*NADPH*	*NADP*$^+$ + *NADPH*
None	5.9	0.15	6.05	1.95	0.0	1.95
Compound S	5.2	0.0	5.2	1.88	0.26	2.14
α-Ketoglutarate	2.2	4.3	6.5	0.16	1.69	1.85
Isocitrate	2.8	3.5	6.3	0.25	2.20	2.45
α-Ketoglutarate + Compound S	1.7	4.1	5.8	0.41	1.87	2.28
Isocitrate + Compound S	2.1	4.3	6.4	0.25	2.12	2.37

tion and utilization of reducing power separately. Therefore, the levels of NADH and NADPH produced as a result of α-ketoglutarate and isocitrate oxidation before and after the addition of Compound S were determined. The results of one representative experiment are illustrated in Table V and clearly indicate that the addition of either α-ketoglutarate or isocitrate causes a reduction of both NAD^+ and $NADP^+$ to almost the same extent. The expected utilization of reducing power (which would be manifested by oxidation of the pyridine nucleotides) in the presence of Compound S was not seen and the direct involvement of either NADH or NADPH in the 11β-hydroxylation of Compound S could not be demonstrated. This was not due to the fact that 11β-hydroxylation of Compound S did not occur because both incubations with α-ketoglutarate and isocitrate in the presence of Compound S led to the formation of 14.7 and 15.1 μg cortisol per mg mitochondrial protein per 15 min, respectively (2.71 and 2.78 mμM cortisol per mg mitochondrial protein per min, respectively). Little cortisol was formed in the absence of α-ketoglutarate or isocitrate (0.01 mμM cortisol per mg mitochondrial protein per min).

It is also interesting to note in passing that, similar to the rat[13], the guinea-pig adrenal mitochondria isolated under our procedure evidently contain insufficient endogenous substrate to keep the pyridine nucleotides in the reduced state. As a result, the mitochondria contain almost exclusively the oxidized forms of NAD^+ and $NADP^+$. The $NAD^+ + NADH/NADP^+ + NADPH$ ratio was found to be 3.15.

The results obtained above indicated, therefore, that reduction of pyridine nucleotides occurred at a faster rate than did their oxidation as a result of their utilization in the 11β-hydroxylation reaction(s). Another series of experiments carried out with the Eppendorf fluorometer served to confirm this. This method, pioneered by ESTABROOK AND MAITRA[28], records in a continuous fashion the changes in levels of reduced pyridine nucleotides (intramitochondrial, in these two instances) by fluorometry. Direct recording of the reduced pyridine nucleotide fluorescence of the mitochondrial suspensions showed that both α-ketoglutarate and isocitrate reduced the pyridine nucleotides to approximately the same extent as recorded by the number of divisions traveled by the pen of the recorder (α-ketoglutarate 168 divisions, isocitrate 175 divisions). The rate of reduction with α-ketoglutarate was faster than with isocitrate (270 sec *vs.* 150 sec). Addition of 330 μM of Compound S altered the pyridine nucleotide steady state very little with both substrates as shown by the deflection (approximately 30 divisions in each case). These results, therefore indicated as in the previous experiments that α-ketoglutarate and isocitrate provide a reducing pressure (leading to reduced pyridine nucleotide accumulation) which is greater than the opposite mechanism directed at releasing this pressure (*i.e.* 11β-hydroxylation leading to oxidized pyridine accumulation).

Figs. 2A and 2B reveal that oxygen uptake was not directly related to cortisol production when α-ketoglutarate and isocitrate were used as oxidizable substrates. These results are not surprising since in the oxidation of isocitrate or α-ketoglutarate O_2 might be expected to be utilized by the respiratory chain as well as the cytochrome P-450 pathway. The reversal of inhibition brought about by addition of isocitrate to the antimycin A blocked α-ketoglutarate system is clearly shown (Fig. 2A). Inhibition of O_2 utilization as well as cortisol production with α-ketoglutarate occurred after addition of 0.50 μg of antimycin A followed by a partial reversal of both these parameters of activity after addition of isocitrate. Isocitrate in these cases could

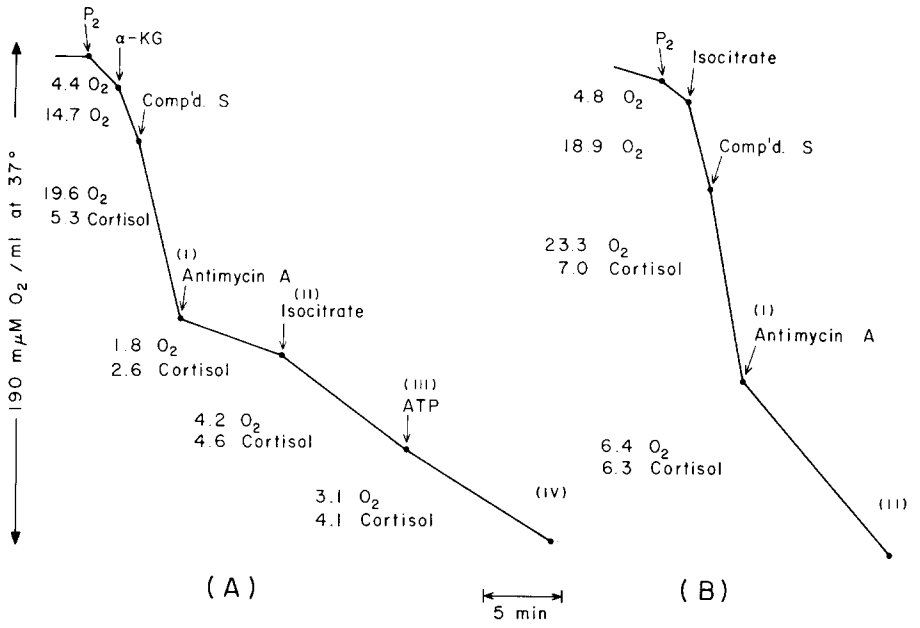

Fig. 2. Guinea-pig adrenal mitochondria P_2 (1.5 mg protein) in 0.20 ml of mixture B, were added to the Gilson oxygraph cell with 0.9 ml usual incubation medium. Further additions were made as shown at arrows (top of curves). Oxygen uptake was measured polarographically using an oscillating platinum electrode at 37°. Suitable aliquots for cortisol determinations were withdrawn from the cell (A) before the addition of (I) antimycin A, (II) isocitrate, (III) ATP, and (IV) about 10 min after ATP; and (B) before the addition of (I) antimycin A and (II) about 10 min after antimycin A. Oxygen uptake and rate of cortisol formation are given below the curves in mμmoles O_2 or cortisol per min per mg mitochondrial protein. α-KG, α-ketoglutarate.

TABLE VI

INHIBITION OF α-KETOGLUTARATE UTILIZATION BY ELECTRON CHAIN INHIBITORS AND BY 2,4-DINITROPHENOL AND REVERSAL OF THIS INHIBITION BY ISOCITRATE

All figures except those designated with asterisks are derived from the same experiment.

Substrate or substance added	*μg cortisol per mg mitochondrial protein*		
	10 mM α-ketoglutarate	*10 mM α-ketoglutarate + 10 mM isocitrate*	*10 mM isocitrate*
120 μg Compound S	46.6	31.0*	35.5*
120 μg Compound S + 1 μg antimycin A	7.7	21.3	33.3
120 μg Compound S + 10 μg oligomycin	34.6	32.0	45.3
120 μg Compound S + amytal, 3 mM	1.3	32.3	41.3
120 μg Compound S + 2,4-dinitrophenol, 100 M	4.7	22.7	38.7
120 μg Compound S + KCN, 3 mM	45.9	43.9	31.0
120 μg Compound S + malonate, 2 mM	30.7	40.0	54.0
120 μg Compound S + Ca^{2+}, 11 mM	6.6	9.3	8.7

* Separate experiment.

maintain a reducing pressure sufficient to account for the conversion of Compound S to cortisol. Thus, the almost stoichiometric relationship of O_2 utilized and cortisol produced, indicated that in the antimycin A blocked system, isocitrate was used for the 11β-hydroxylation of Compound S by making NADPH available, and did not appear to require activity of the respiratory chain. The data of Table VI serve to confirm those presented in Table II, as well as supporting the aforementioned oxygraph findings. In all cases, α-ketoglutarate utilization was inhibited by the inhibitors of the electron-chain (except KCN) as well as the uncoupler 2,4-dinitrophenol. Oligomycin addition, even at the high level of 10 μg/ml of incubation medium (10 μg/1.5 mg mitochondrial protein), had only a small inhibitory effect. The addition of isocitrate in these incubations, as in the oxygraph studies, reversed to a large extent the α-ketoglutarate blocked system. As was found with rat adrenal mitochondria incubated in the presence of several Krebs-cycle intermediates, the addition of 11 mM Ca^{2+} to guinea-pig adrenal mitochondria inhibited the Compound S to cortisol reaction when isocitrate and α-ketoglutarate were used as the source of oxidizable substrates. On the other hand, malonate had little effect on α-ketoglutarate utilization when added in a concentration of inhibitor to substrate ratio of 1:5.

DISCUSSION

In this study there is little doubt that exogenously added NADPH was utilized for 11β-hydroxylation of Compound S and deoxycorticosterone in guinea-pig adrenal mitochondria when fairly high levels of Ca^{2+} were present in the incubation medium. This finding confirms well-tabulated data published in the last few years and obtained with rat adrenal mitochondria, which showed that Ca^{2+} (refs. 12, 14–16) as well as other agents[15,29] which bring about swelling of the mitochondria probably act by making NADPH available to the factors involved in the 11β-hydroxylation reaction. By analogy with the findings in rat adrenal mitochondria and because swelling was also observed with the guinea-pig mitochondria (Fig. 1) it can be concluded that NADPH is oxidized *via* a NADPH dehydrogenase (flavoprotein–non-heme iron component) and there results a sequence of reactions leading to electron transport which is associated with the transfer of reducing equivalents (from NADPH) to cytochrome P-450. Another point of similarity between the behavior of rat adrenal mitochondria and the guinea-pig mitochondria toward exogenous NADPH and Ca^{2+} also lies in their being inhibited by ferricyanide.

The finding of isocitrate action being unaffected by the classical electron chain inhibitors, antimycin A and cyanide, presents evidence to support the conclusion that oxidation of isocitrate for steroid 11β-hydroxylation occurs independently of its oxidation by the classical electron chain. The incubations with isocitrate which led to the formation of endogenous NADH (see Table V) demonstrate that guinea-pig adrenal mitochondria like rat adrenal mitochondria have either two isocitric acid dehydrogenase enzymes (one being NAD^+- and the other $NADP^+$-linked) or one isocitrate dehydrogenase enzyme with dual pyridine nucleotide specificity. In any case, because of the lack of inhibition observed with antimycin A, cyanide and the small inhibition in 11β-hydroxylation brought about by the uncoupler 2,4-dinitrophenol it would appear that the endogenous NADPH is the co-factor used for steroid hydroxyla-

tion in these cases. Production of NADPH as a result of oxidation of the NADH produced by isocitrate oxidation would appear unlikely in an antimycin A-blocked preparation because of the energy requirements of the energy-linked transhydrogenase[31–32].

Although oxidation of α-ketoglutarate gave rise to reducing equivalents in the form NADPH and NADH (Table IV), it would appear that utilization of this substrate is much more dependent on activity of the classical electron chain because both antimycin A and 2,4-dinitrophenol had pronounced inhibitory effects. Thus, it is quite plausible that α-ketoglutarate gave rise to NADPH as a result of oxidation of reduced NADH *via* the energy-requiring transhydrogenase enzyme. Perplexing, however, was the finding that cyanide, known to block succinate and malate utilization in the rat adrenal mitochondria[12,14], did not block the 11β-hydroxylation of Compound S. At the moment, we have no explanation for this finding except to suggest the remote possibility that the mitochondria were not permeable to cyanide and thus did not inhibit cytochrome oxidase.

The results of the oxygraph studies serve to reinforce the above findings and conclusions. A clear delineation of α-ketoglutarate and isocitrate action was recorded both with respect to O_2 uptake and Compound S converted into cortisol in the antimycin A inhibited system. The stoichiometry achieved in O_2 uptake and cortisol biosynthesis in the aforementioned system, indicates that little production of other oxygenated steroids occurred. This agrees with our paper chromatographic findings which revealed that little or no 18-hydroxylated or more highly oxygenated steroids than cortisol were synthesized from Compound S.

The above findings with NADPH + Ca^{2+} and isocitrate have bearing on the conclusions reached by SPATZ AND HOFMANN[19]. It can now be said unequivocally that guinea-pig adrenal mitochondria utilize exogenous NADPH efficiently. Our finding with isocitrate makes it likely that oxidation of this substrate also brings about intramitochondrial reduction of NADPH which then becomes available to the members of the P-450 chain for 11β-hydroxylation. Although more work needs to be done in delineating the possible pathways of α-ketoglutarate oxidation and the function of the electron chain in this respect, it is indeed possible that NADPH is made available as a result of NADH oxidation. As mentioned above, at the moment we have no explanation why succinate and malate are not used as substrates whereas in SPATZ AND HOFMANN'S[19] studies it was found that succinate, fumarate and α-ketoglutarate were capable of supporting 11β-hydroxylation of Compound S equally well. It is to be noted that in our experiments, utilization of succinate and malate was made possible by additions of ATP and led to a small but significant amount of cortisol biosynthesis (Table II).

Another finding which stands out and is different from that reported by SPATZ AND HOFMANN, is that our mitochondrial preparations (P_2) had little or no 21-hydroxylating capacity when incubated with progesterone. The only product formed was 11β-hydroxyprogesterone. The similarity of these results with those obtained in the rat[12–16] led us to conclude that our fractionation procedure did indeed yield a mitochondrial preparation uncontaminated with the microsomal fraction. In the rat preparations, electron microscopic evidence indicated a good mitochondrial preparation[16,33] when our fractionation procedure was used. It might be of value to mention also, that on several occasions, upon checking for respiratory control in guinea-pig

adrenal mitochondria, a P/O ratio of 2.8 was obtained when α-ketoglutarate was used as oxidizable substrate. The ratio of 2.8 (theoretically possible 4) can be considered sufficiently high to indicate biochemical integrity of a tissue fraction considered for the most part mitochondrial in nature.

That the distribution of steroid hydroxylases and their association with different particulate fractions do not appear to be different in the guinea-pig or rat adrenal also is evident from data shown in Table III. The incubation of P_4 (microsomes) with pregnenolone and progesterone led to their conversion into Compound S. Little or no steroid substrate conversion occurred in the absence of NADPH and the addition of 11 mM Ca^{2+} had no effect at this level. Also little 11β-hydroxylation of deoxycorticosterone and Compound S into corticosterone and cortisol, respectively, was found.

The addition of the 103000 × *g* supernatant fraction to the microsomes led to the production of Compound S from pregnenolone and progesterone whereas deoxycorticosterone and Compound S were not 11β-hydroxylated in significant amounts. This occurred in the absence of NADPH additions. Presumably, the high-speed supernatant contains sufficient NADPH to supply the microsomal system involved in the steroid hydroxylations. In this instance, this finding is somewhat different from that in the rat where it was found that pregnenolone or progesterone was not transformed in the P_4 + high-speed supernatant fraction combinations unless isocitrate or succinate were also added to the system.

The addition of isocitrate to P_4 + high-speed supernatant fractions did lead to substantial increases in transformation of pregnenolone and progesterone into Compound S showing that isocitrate can be utilized to support NADPH-dependent steroid hydroxylations occurring in the microsomes. Presumably the high-speed supernatant fraction or the microsomes contain $NADP^+$-linked dehydrogenases required for oxidation of isocitrate and thus NADPH is formed.

The salient point derived from these latter studies was that we could obtain guinea-pig microsomes which were presumably uncontaminated from mitochondria. This was assessed by their capacity to 21-hydroxylate progesterone without converting deoxycorticosterone or Compound S into corticosterone or cortisol.

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REFERENCES

1 B. W. HARDING, S. H. WONG AND D. H. NELSON, *Biochim. Biophys. Acta*, 92 (1964) 415.
2 T. OMURA, R. SATO, D. Y. COOPER, O. ROSENTHAL AND R. W. ESTABROOK, *Federation Proc.*, 24 (1965) 1181. (Symposia.)
3 B. W. HARDING, L. D. WILSON, S. H. WONG AND D. H. NELSON, *Steroids*, Suppl. II (1965) 51.
4 T. KIMURA AND K. SUZUKI, *J. Biol. Chem.*, 242 (1967) 485.
5 B. W. HARDING AND D. H. NELSON, *J. Biol. Chem.*, 241 (1966) 2212.
6 S. NARASIMHULU, D. Y. COOPER AND O. ROSENTHAL, *Life Sciences*, 4 (1965) 2101.
7 D. Y. COOPER, S. NARASIMHULU, A. SLADE, W. RAICH, O. FOROFF AND O. ROSENTHAL, *Life Sciences*, 4 (1965) 2109.

8 K. SUSUKI AND T. KIMURA, *Biochem. Biophys. Res. Commun.*, 19 (1965) 340.
9 S. HORIE, T. KINOSHITA AND N. SHIMAZONO, *J. Biochem.*, 60 (1966) 660.
10 R. W. ESTABROOK, D. Y. COOPER AND O. ROSENTHAL, *Biochem. Z.*, 338 (1963) 741.
11 S. OLDHAM, D. H. NELSON AND B. W. HARDING, *Federation Proc.*, 25 (1966) 551.
12 F. GUERRA, F. G. PÉRON AND J. L. MCCARTHY, *Biochim. Biophys. Acta*, 117 (1966) 433.
13 J. PURVIS, R. BATTU AND F. G. PÉRON, in K. MCKERNS, *Function of Adrenal Cortex*, Appleton-Century-Crofts, 1967, in the press.
14 F. G. PÉRON AND J. MCCARTHY, in K. MCKERNS, *Function of the Adrenal Cortex*, Appleton-Century-Crofts, 1967, in the press.
15 F. G. PÉRON, F. GUERRA AND J. L. MCCARTHY, *Biochim. Biophys. Acta*, 110 (1965) 277.
16 F. G. PÉRON, J. L. MCCARTHY AND F. GUERRA, *Biochim. Biophys. Acta*, 110 (1965) 277.
17 J. L. MCCARTHY AND F. G. PÉRON, *Biochemistry*, 6 (1967) 25.
18 L. SPATZ AND F. G. HOFMANN, *Federation Proc.*, 24 (1965) 449.
19 L. SPATZ AND F. G. HOFMANN, *Endocrinology*, 79 (1966) 971.
20 R. H. SILBER, R. D. BUSCH AND R. OSLAPAS, *Clin. Chem.*, 4 (1958) 278.
21 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
22 F. MONCLOA, F. G. PÉRON AND R. I. DORFMAN, *Endocrinology*, 65 (1959) 717.
23 S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 895.
24 J. L. PURVIS, *Biochim. Biophys. Acta*, 38 (1960) 435.
25 I. E. BUSH, *Biochem. J.*, 50 (1952) 370.
26 F. H. ELLIOTT, M. K. BIRMINGHAM, A. V. SCHALLY AND E. SCHÖNBAUM, *Endocrinology*, 55 (1954) 271.
27 F. G. PÉRON AND R. I. DORFMAN, *Methods in Hormone Research*, Academic Press, New York, 1962, p. 199.
28 R. W. ESTABROOK AND P. K. MAITRA, *Anal. Biochem.*, 3 (1962) 369.
29 I. N. HIRSCHFIELD AND S. B. KORITZ, *Biochemistry*, 12 (1964) 1994.
30 L. DANIELSON AND L. ERNSTER, *Biochem. Biophys. Res. Commun.*, 10 (1963) 91.
31 C. P. LEE AND L. ERNSTER, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Process in Mitochondria*, Elsevier, 7 (1966) 218.
32 L. DANIELSON AND L. ERNSTER, in B. CHANCE, *Energy-Linked Function of Mitochondria (1st Coll. Johnson Res. Foundation)*, 1 (1963) 157.
33 F. G. PÉRON, F. GUERRA AND J. L. MCCARTHY, *Federation Proc.*, 24 (1965) 449.