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ACIDIC STEROID METABOLITES: BIOSYNTHESIS OF STEROID CARBOXYLIC ACIDS BY RABBIT LIVER MICROSOMES AND THE INVOLVEMENT OF A CYTOCHROME P_{450}-INDEPENDENT MIXED FUNCTION OXIDASE SYSTEM

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SUMMARY

[1,2-^{3}H]-4-pregnene-3,20-dione-21-oic acid (pregnenoic acid*), and [1,2-^{3}H]-4-androsten-3-one-17β-carboxylic acid (etienic acid) have been isolated and characterised as *in vitro* metabolites of progesterone and deoxycorticosterone after incubation with rabbit liver microsomes. Acid formation required aerobic conditions and NADPH. It was not inhibited by carbon monoxide nor stimulated by pre-treatment of the live rabbit with sodium phenobarbital; but was inhibited by the presence of cyanide. These properties indicate the oxidation of P and DOC to C-21-steroidal carboxylic acids by the rabbit hepatic microsomal system is catalysed by a cyanide sensitive-mixed function oxidase that is independent of cytochrome P_{450}.

INTRODUCTION

In vivo studies have indicated that a major metabolic pathway of progesterone in the New Zealand White rabbit involves the oxidation of the acetyl side-chain to a 20-oxo-21-oic acid function and the excretion of these steroid acids in the urine [1, 2].

A previous *in vitro* study noted that rabbit liver microsomes were a major site for the oxidation of both [^{3}H]-P and [^{3}H]-DOC to steroid acids, the structures of which were not determined [3].

The present study extends these observations to the identification of the acidic metabolites formed *in vitro* and the partial characterisation of the oxidative enzyme system involved as a cytochrome P_{450}-independent mixed function oxidase.

METHODOLOGY

Standard incubation conditions

Rabbit liver microsomes or mitochondria were prepared from female New Zealand White rabbits (4–5 kg) as previously described [3]. Incubations with either [1,2-^{3}H]-progesterone (55.7 Ci/mmol) or [1,2-^{3}H]-deoxycorticosterone (46.8 Ci/mmol) (New England Nuclear, Canada, Ltd.) were carried out with a protein:substrate ratio of 20,000:1 in the presence of a NADPH generating system unless otherwise stated. The latter was prepared by adding NADP (45 ng), glucose-6-phosphate (0.28 μg) and glucose-6-phosphate dehydrogenase (5×10^{-5} U) per ng substrate to each incubation. Substrates were added in propylene glycol (0.1 ml) solution and incubations were carried out for 2 h at 37°C in an atmosphere of air in a shaking water bath. Reactions were terminated by the addition of ethyl acetate (5 ml) and the incubates were extracted with the same solvent at pH 1 and partitioned into neutral and acidic metabolite fractions as previously described [3].

Steroid acid standards

Pregnenoic acid was synthesised by the oxidation of DOC to the 21-dehydro DOC with cupric acetate (4) and further oxidation of the aldehyde with chromium trioxide in the presence of NaCN, as described by Monder (5).

Yield 42.8%; m.p. 184–186° (Literature 181–184° (dec.) (5); 183–186°) (11). Etienic acid was purchased from Steraloids, U.S.A.

Steroid blocking experiments

Incubations of [^{3}H]-progesterone ([^{3}H]-P) and [^{3}H]-deoxycorticosterone ([^{3}H]-DOC) with hepatic microsomes were carried out in the presence of either pregnenoic or etienic acids which were added at the rate of 500 μg/incubate. The acidic metabolites were isolated in the usual way and the appropriate steroid acids were detected by U.V. light absorption after t.l.c. and the specific activities (d.p.m./μg) were calculated by measuring their absorptions at 240 nm with a Unicam SP 500 Spectrophotometer.

* The following trivial names and abbreviations have been used: P, progesterone (4-pregnene-3,20-dione); DOC, deoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione); pregnenoic acid (4-pregnene-3,20-dione-21-oic acid); etienic acid (4-androsten-3-one-17β-carboxylic acid); 21-dehydro DOC (4-pregnene-3,20-dione-21-al).

Variations in standard incubation conditions

Anaerobic experiments. The complete incubation mixtures were either gassed directly with nitrogen for 2 min and incubated or glucose oxidase (1000 U) and glucose (30 mg) were first added, then the mixture was gassed with nitrogen. Incubations were carried out in screw capped vials which were flushed with nitrogen before sealing.

Carbon monoxide experiments. Incubates were gassed by bubbling a carbon monoxide–air (90:10, v/v; Canadian Liquid Air) mixture through for 5 min, and then were incubated either in the dark or light in the usual way.

Sodium phenobarbital stimulation experiments

Rabbits were injected i.p. with a solution of sodium phenobarbital (40 mg/kg) in 0.9% saline (1.5 ml) on four consecutive days [6]. The livers were removed 24 h after the last injection and processed for the microsomes and mitochondria in the usual way.

Identification of steroid metabolites

Mitochondria and microsomes prepared from phenobarbital-treated and control rabbit livers were incubated with [^{3}H]-P, and [^{3}H]-DOC was isolated from the neutral fractions and quantitated as previously described [7].

Steroid acids were analysed by sequential t.l.c. on silica gel GF_{254} coated plates (0.25 mm thickness) in the following systems: System 1. Ethyl acetate–formic acid (99:1, v/v). System 2. Chloroform–isopropyl alcohol–formic acid (97:2:1, by vol.) System 3. Chloroform–formic acid (99:1, v/v). Non-labelled standards were detected by their U.V. absorption at 240 nm and labelled metabolites with a radiochromatogram scanner (Packard Model 7201).

Non-labelled pregnenoic and etienic acids (20 mg each) were added to the appropriate radioactive fractions and each was crystallised to constant specific activity (d.p.m. ^{3}H/mg) after methylation. Radioactivity was measured in a PCS–Xylene (1:1, v/v) scintillation mixture (Amersham-Searle, U.S.A.) and counting efficiency for tritium (30–35%) was determined with the automatic external standard of a Packard Liquid Scintillation Counter (Model 3365).

RESULTS

Conditions for the formation of acidic metabolites of [1,2-^{3}H]-progesterone and [1,2-^{3}H]-deoxycorticosterone by rabbit liver microsomes

Microsomal protein:substrate ratio. Table 1 gives the recovery of radioactivity in the acidic fractions when [^{3}H]-P or [^{3}H]-DOC were incubated with rabbit liver microsomes at different protein:substrate ratios. Acid formation increased with both steroids up to a 20,000:1 ratio, which was maximal for [^{3}H]-DOC. [^{3}H]-P gave a further small increased yield of acids at 50,000:1.

Effect of steroid vehicle on the formation of progesterone metabolites. The effect of the addition of [^{3}H]-P in either propylene glycol or ethanol on the formation of metabolite fractions by liver microsomes is given in Table 2. The distribution of radiometabolites between the free neutral, conjugated and acidic fractions was assessed by solvent partition after appropriate hydrolyses [9]. Propylene glycol (0.1 ml) was a more effective solvent for the formation of acids than ethanol. It also has an inhibitory action on the formation of steroid glucuronides, a property that has been previously reported [1].

Specificity of co-factors for acid formation. Table 3 gives the formation of acids when [^{3}H]-P and [^{3}H]-DOC were incubated with microsomes in the presence of reduced and oxidised co-factors. The highest yields of acids were obtained with both steroids incubated in the presence of NADPH, preferably generated *in situ*. The other co-factors gave low yields of acids with [^{3}H]-P and the yield of acids from [^{3}H]-DOC was reduced to approximately one third of that with NADPH.

Optimum pH of incubation medium for acid formation. The effect of the pH of the sodium phosphate (0.2 M) incubation medium on the *in vitro* yield of acidic metabolites of [^{3}H]-P and [^{3}H]-DOC is shown in Fig. 1. Although the yield of acids was lower with this microsomal preparation than with previous experiments, a pH optimum at pH 7.4 is clearly defined with both substrates.

Effect of different experimental conditions on acid formation

Formation of acidic metabolites of [^{3}H]-P and [^{3}H]-DOC by rabbit liver microsomes required aerobic conditions. Replacement of air by either bubbling nitrogen through the incubation medium for 5 min, or by pre-incubation with glucose oxidase, followed by saturation with nitrogen, inhibited acid formation by an average of 72.2% and 75.5% respectively. By contrast, saturation with a carbon monoxide in air mixture (90:10, v/v) and incubation in the light or

Table 1. Effect of microsomal protein:substrate ratio on the formation of acidic metabolites

Protein*:substrate ratio ($\times 10^3$)	% Formation of acids	
	[^{3}H]-P	[^{3}H]-DOC
0.5	2.6	3.9
1.0	6.1	8.3
10.0	24.1	26.9
20.0	27.1	35.3
30.0	26.9	30.5
40.0	26.5	34.1
50.0	29.9	29.8
100.0	27.9	31.9
500.0	29.5	25.1

* Microsomal protein determined by the method of Lowry *et al.*[8]. [^{3}H]-P (1.7 ng) and [^{3}H]-DOC (0.97 ng) incubated under standard conditions.

Table 2. Effect of steroid vehicle on the distribution of radioactivity between the metabolite fractions of [^{3}H]-P after incubation with hepatic microsomes

Steroid	% Distribution of radioactivity		
	Propylene glycol (0.1 ml)	Ethanol (0.1 ml)	Propylene glycol (0.1) + Boiled control
Neutral metabolites			
Free	65.8	45.1	80.1
Glucuronides	4.7	25.7	3.1
Sulfates	1.5	0.5	1.3
Acidic metabolites	27.4	16.8	2.6

Mean of duplicate experiments carried out under standard conditions.

dark, failed to significantly influence acid formation; inhibition averaged 5.1% and 3.4% with [^{3}H]-P and [^{3}H]-DOC as substrate. Evidence for a cyanide sensitive component in the formation of acids from [^{3}H]-DOC was indicated by 67.1% inhibition of acid formation with incubations carried out in the presence of cyanide ion (0.1 mM).

Effect of the administration of sodium phenobarbital to the live rabbit on the in vitro *acid formation by rabbit liver microsomes.* Hepatic microsomes and mitochondrial, prepared from control and sodium phenobarbital treated rabbits as described in the Methods, were incubated with [^{3}H]-P and [^{3}H]-DOC and the formation of acids compared. Table 4 gives the yield of acids when [^{3}H]-P was incubated. No significant stimulation in acid formation from [^{3}H]-P or [^{3}H]-DOC was observed with the phenobarbital treated rabbits. Lack of significant stimulation of the 21-hydroxylation of [^{3}H]-P by the preparations from phenobarbital treated rabbits was also indicated when the yield of [^{3}H]-DOC was compared to that of the control animals (Table 4).

Identification of the acidic microsomal metabolites of tritiated progesterone and deoxycorticosterone

The acidic metabolites of [^{3}H]-P and [^{3}H]-DOC isolated from incubations with rabbit liver microsomes under the standard conditions given in the Methods were analysed by t.l.c. Two major radioactive peaks, corresponding to the standard pregnenoic acid and etienic acid were isolated and further characterised as indicated below.

Identification of [1,2-^{3}H]-4-pregnene-3,20-dione-21-oic acid. The major acidic radiometabolite of [^{3}H]-P and [^{3}H]-DOC had an R_f value of 0.35 in System 1, corresponding with the U.V. absorption area of co-chromatographed pregnenoic acid. This represented 30.2% and 46.2% of the [^{3}H]-P and [^{3}H]-DOC acids, increasing to 53.5% and 68.6% respectively when incubations were carried out in the presence of pregnenoic acid (500 μg) as a metabolic blocking agent.

The isolated [^{3}H]-pregnenoic acid was further purified by chromatography in System 2 (R_f p.53) and was characterised by crystallisation to constant specific activity with the authentic standard (Table 5).

Non-labelled DOC (1 mg) plus [^{3}H]-DOC was also incubated under standard conditions with rabbit liver microsomes at a protein:substrate ratio of 2000:1 and the pregnenoic acid zone was isolated by t.l.c. in System 1 and methylated. GLC gave a single peak (r 0.59) which corresponded with the mobility of synthetic pregnenoic acid methyl ester when run singly and

Table 3. Effect of co-factors on the yield of acidic metabolites

Co-factor (0.6 μmol)	% Formation of acids	
	[^{3}H]-P	[^{3}H]-DOC
NAD^+	3.0	11.4
$NADP^+$	4.0	9.8
NADH	3.7	9.6
NADPH	23.3	26.8
NADPH-generating* System	28.7	31.6

* $NADP^+$ (0.6 μmol); glucose-6-phosphate (0.1 mol); glucose-6-phosphate dehydrogenase (0.5 U).

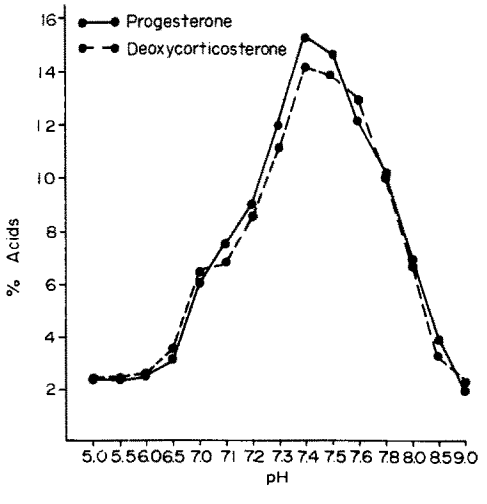


Fig. 1. Effect of pH of incubation medium on the formation of steroid acids by rabbit liver microsomes.

Table 4. Effect of sodium phenobarbital treatment of rabbits on the *in vitro* formation of acidic metabolites and the 21-hydroxylation of [^{3}H]-P by hepatic microsomes and mitochondria

Incubation period (min)	Acidic metabolites (percentage formation)				Neutral metabolites (pmol DOC/mg protein*)			
	Microsomes		Mitochondria		Microsomes		Mitochondria	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
15	9.7	10.1	3.5	4.4	6.6	7.5	9.6	10.0
30	25.8	29.3	5.4	5.9	22.5	25.2	25.9	26.3
60	22.4	23.4	4.8	7.3	21.3	25.1	24.7	25.0
120	19.6	23.0	5.0	6.9	11.4	15.0	17.8	18.1

* Isolated and quantitated as previously described [7].

admixed on a 3% SE-30 column (240°C; progesterone internal standard).

Identification of [1,2-^{3}H]-4-androstene-17β-carboxylic acid. The second major acidic metabolite of [^{3}H]-P and [^{3}H]-DOC had R_f values of 0.76 and 0.53 in t.l.c. Systems 1 and 3 respectively and was characterised as etienic acid (Table 6) by a similar series of reactions as described for pregnenoic acid. The proportions of the etienic acid isolated often showed wide variations and it was uncertain whether this was due to variations in enzymatic activity or artefactual breakdown. That the latter occurs to some degree was observed during the purification of synthesised pregnenoic acid. [^{3}H]-P invariably gave greater yields of etienic acid than [^{3}H]-DOC when incubated with rabbit liver microsomes and the yield also increased when incubations were carried out in the presence of synthetic etienic acid (500 μg) as a blocking agent.

Several minor polar acids that appear to be 6-hydroxylated metabolites of pregnenoic acid and etienic acid were also detected but not characterised.

Table 5. Characterisation of [^{3}H]-pregnenoic acid as a metabolite of [^{3}H]-P and [^{3}H]-DOC

Treatment	[^{3}H]-P metabolite		[^{3}H]-DOC metabolite	
	No blocker	Blocker*	No blocker	Blocker
t.l.c. System 1.	67,000 d.p.m.	251 d.p.m./μg	86,400 d.p.m.	365 d.p.m./μg†
t.l.c. System 2.	66,700 d.p.m.	252 d.p.m./μg	85,190 d.p.m.	369 d.p.m./μg
Pregnenoic acid added: Calculated SA (d.p.m./mg)	20 mg 3335	20 mg 5417	20 mg 4259	20 mg 16,063
Crystallisation from acidified $NaHCO_3$ soln (d.p.m./mg)	XL 3336 ML 3129	5418 5303	4262 3259	16,055 15,055
Methylation: crystallisation from aqueous methanol (d.p.m./mg)	XL 3339 ML 3327	5431 5401	4265 4257	16,067 16,051

* Incubated with 500 μg pregnenoic acid.
† Calculated from O.D. at 240 nm. XL = crystals; ML = mother liquor

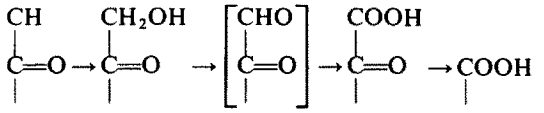
Table 6. Characterisation of [^{3}H]-etienic acid as a metabolite of [^{3}H]-P

Treatment	No blocker	Blocker*
t.l.c. System 1.	29,600 d.p.m.	195 d.p.m./μg†
t.l.c. System 3.	28,130 d.p.m.	139 d.p.m./μg
Etienic acid added: Calculated SA (d.p.m./mg)	20 mg 1407	20 mg 3215
Crystallisation from acidified $NaHCO_3$ soln. (d.p.m./mg)	XL 1407 ML 1108	3218 3006
Methylation; Crystallisation from aqueous methanol (d.p.m./mg)	XL 1415 ML 1405	3222 3213

* Incubated with 500 μg etienic acid.
† Calculated from O.D. at 240 nm. XL = crystals; ML = mother liquor.

DISCUSSION

The present studies on the oxidative metabolism of P and DOC by rabbit liver microsomes indicate that the isolated preparations have the capacity to oxidise the acetyl side-chain of P to the 21-oic acid of pregnenoic acid, and possibly further to the C-20-etienic acid. 21-Hydroxylation of P to DOC by rabbit liver microsomes has previously been demonstrated [7] so it is now possible to formulate a simple oxidative pathway as follows:



Oxidation of the α-ketol side-chain is presumed to proceed via the 21-aldehyde. Though this compound has not been isolated as an intermediate, it has recently been shown to be oxidised to a 21-oic acid by rabbit liver microsomes (unpublished observation).

The major acidic metabolites of P isolated from rabbit urine [2] and from *in vitro* liver microsomal incubates, are predominantly 20-oxo-21-oic acids. 20-Hydroxylated acids have not been identified as *in vitro* metabolites and form only a minor fraction of the acidic urinary metabolites of P [2]. Further oxidation of the acidic side-chain to the C-20-etienic acid appears to be an enzymatic property of the isolated microsomes, though some side-chain cleavage of 20-oxo-21-oic acids has been detected during handling. Etienic acids have also been detected as metabolites of P excreted in rabbit urine, though they were quantitatively less significant than the 21-oic acids [2]. Several mammalian species have also been shown to form minor quantities of etienic acids as metabolites of DOC, as recently reviewed by Monder and Bradlow[12].

The enzyme system of rabbit liver microsomes that oxidises the 17β-acetyl and α-ketol side-chains of P and DOC respectively appears to be unique in being the only membrane bound enzyme system identified so far with the formation of carboxylic acid metabolites of steroids. The oxidation of the alkyl side-chains of aromatic compounds by rabbit liver microsomes resembles that of P in that the first step is a NADPH-dependent hydroxylation, but differs in that further oxidation to the carboxylic acid requires a NAD-dependent enzyme system that is localised in the soluble fraction of the liver [6, 13]. The enzymes that oxidise DOC and cortisol to 21-oic acids are also localised in the soluble fractions of human [14] and hamster [15] livers.

NADPH and aerobic incubation conditions are required for the *in vitro* oxidation of DOC to a 21-oic acid by rabbit liver microsomes, and presumably also for the 21-hydroxylation of P to DOC since acid formation from P also requires the same conditions. These properties are characteristic of a mixed function oxidase [16]. Mixed function oxidases are typically associated with cytochrome P_{450}, a carbon monoxide-binding cytochrome [17] that is abundant in hepatic microsomes, where it has been implicated in the metabolism of drugs and xenobiotics [18], and in the mitochondria and microsomes of the adrenal cortex, where it functions in the hydroxylation of steroids [19]. The failure to inhibit acid formation by rabbit liver microsomes with carbon monoxide and the lack of significant stimulation when sodium phenobarbital was administered to the live rabbit implies that the mixed function oxidation of P and DOC is catalysed by a microsomal enzyme system that does not require cytochrome P_{450}.

The methyl sterol oxidase system of rat liver microsomes that has been extensively studied by Gaylor and co-workers [20–22] bears a number of resemblances to the rabbit liver microsomal system currently under study. Thus, the oxidation of the C–4 methyl groups of lanosterol follows a similar pathway of hydroxylation and oxidation to carboxylic acids. The reactions are also catalysed by a mixed function oxidase that is independent of cytochrome P_{450}, but is inhibited by cyanide. Cytochrome P_{450}-independent mixed function oxidases have also been implicated in two further systems, namely; the desaturation of fatty acyl CoA esters [23] and the biosynthesis of plasmalogens from alkanyl ethers [24]

The evidence reported here for the involvement of a cytochrome P_{450}-independent mixed function oxidase in the oxidative formation of steroid carboxylic acids by rabbit liver microsomes is the first implication of such a system in steroid metabolism.

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