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ACIDIC STEROID METABOLITES: *IN VITRO* METABOLISM OF TRITIATED PROGESTERONE AND DEOXYCORTICOSTERONE BY RABBIT LIVER

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SUMMARY

Rabbit liver subcellular fractions incubated *in vitro* with tritiated progesterone or deoxycorticosterone gave neutral and acidic metabolites. Deoxycorticosterone formed acids at a faster rate, and gave a greater yield (41·5% ± 1·8 S.D.) than progesterone (36·6% ± 2·6 S.D.) at 120 min incubation with microsomal-supernatant (10,000 *g*) preparations. These results indicate that while a C-21-hydroxyl function facilitates acid formation, its absence does not preclude it. Acid formation by subcellular fractions was localised in the microsomal fraction, with low activity in the cytosol and negligible activity in the mitochondrial fractions. This is the first reported *in vitro* formation of acidic steroid metabolites by rabbit liver.

INTRODUCTION

The *in vitro* metabolism of progesterone has been studied with rabbit liver [1, 2], kidney [3], mammary gland [4] and epididymis [5], and the formation of neutral metabolites extensively catalogued. No acidic metabolites were identified in these studies, attention being directed primarily to the purification of neutral metabolites prior to identification. With the recent demonstration that a major metabolic pathway of progesterone in the rabbit leads to the urinary excretion of metabolites with a C-21-carboxylic acid function [6] it was of interest to re-examine the *in vitro* metabolism of progesterone with rabbit liver to resolve the anomalous situation of acidic metabolites being identified as products of *in vivo* [7–9] but not *in vitro* metabolism.

Deoxycorticosterone was also incubated to determine the effect of a C-21-hydroxyl function on the formation of acids. The *in vitro* formation of neutral metabolites of deoxycorticosterone by rabbit liver [10], and C-21-carboxylic acids by guinea pig liver [11] have been reported. Steroid carboxylic acids have also been identified as metabolic products of 21-dehydrocorticosterone with sheep liver supernatant *in vitro* [12] and as urinary metabolites of fluocortolone [13], cortisol [14, 15] and 21-dehydrocortisol [16] in the human.

MATERIALS AND SUPPLIES

Solvents, chemicals and radioisotopes were obtained from the same sources, and radioisotopes counted under the same conditions are previously described [17]. Purity of [1,2-^{3}H]-progesterone (51 Ci/mmol) and [1,2-^{3}H]-deoxycorticosterone (30 Ci/mmol) was assessed to be satisfactory as purchased, by t.l.c. on silica gel GF_{254} (0·25 mm) in the system chloroform–ethyl acetate (9:1, v/v) and radiochromatogram scan.

METHODS

Preparation of liver subcellular fractions. Female New Zealand white rabbits were sacrificed with a blow on the neck. The liver was rinsed in ice-cold 0·15 M KCl solution, blotted, and a 20% w/v homogenate prepared with a Potter–Elvjhem glass homogenizer and Teflon pestle (8 strokes). The homogenate medium and centrifugation conditions were as reported by Cooke and Vallance [18] to give microsomal–supernatant (M–S) and mitochondrial fractions (10,000 *g*). High speed centrifugation (105,000 *g*) of the M–S fraction gave microsomal and cytosol fractions. The microsomal pellet and cytosol fractions were purified further by an additional centrifugation at 105,000 *g* (1 h), the former being resuspended in homogenate buffer.

Microsomal and mitochondrial pellets were resuspended in homogenate buffer and stored at −17°C until incubated.

Incubation. Tritiated progesterone and deoxycorticosterone were dissolved in propylene glycol (0·2 ml) and incubated with either liver slices (50–500 mg) or subcellular fractions (0·2–3·0 ml). NADP (0·6 μmol), glucose-6-phosphate (0·1 M) and glucose-6-phosphate dehydrogenase (0·5 Units) were added to some incubates and the mixtures shaken in a metabolic incubator at 37°C for 15–120 min in an atmosphere of air. The reaction was terminated by addition of acetone (1 ml).

The following trivial names and abbreviations are used: progesterone (P); 4-pregnene-3,20-dione; deoxycorticosterone (DOC); 21-hydroxy-4-pregnene-3,20-dione.

Hydrolysis and partition. Incubates were saturated with NaCl, adjusted to pH 1 with HCl, extracted with ethyl acetate (3 × 2 vol.) and an aliquot removed for counting. Recovery of radioactivity averaged 90%. The extracts were hydrolyzed with either Glusulase (0·1 ml containing 13,950 Units β-glucuronidase; 4300 Units sulfatase; Endo Laboratories, U.S.A.), in sodium acetate buffer (0·1 M; pH 5·0) for 24 h at 37°C, or with 15% HCl (w/v) for 20 min in a boiling water bath. Metabolites were partitioned between ethyl acetate and 0·1 M sodium bicarbonate solution to give neutral and acidic fractions respectively. Acids were re-extracted into ethyl acetate after acidification.

Protein determination. Protein concentrations in subcellular fractions were measured by the Lowry method [19].

Methylation. Acidic fractions were methylated with boron trifluoride in methanol (14% w/v); Sigma Chemical Co., U.S.A.) [20] and the neutral methyl esters extracted into ethyl acetate from alkaline solution.

RESULTS

Formation of acidic steroid metabolites with rabbit liver slices. In a preliminary experiment rabbit liver slices of different weights were incubated with tritiated P and DOC, with and without the addition of a NADPH-generating system. Table 1 indicates a significant stimulation of acid formation with addition of co-factors up to a maximum of 12·2% (P) and 16·3% (DOC).

Formation of acids with microsomal–supernatant incubations. Increased formation of acids was obtained with M–S (10,000 *g*) preparations of rabbit liver incubated in the presence of co-factors. Table 2 compares the recovery of neutral and acidic metabolites at different incubation times. DOC was metabolized to acids at a significantly faster rate than P and gave a maximal yield of 41·5% at 120 min incubation compared to 36·6% for P. The boiled control did not give significant acids.

The neutral and acidic metabolites were estimated after hydrolysis with 15% HCl on a boiling water bath (20 min), which was the most convenient and expedient method. Table 3 compares the yield of acids obtained after enzyme (Glusulase) and hot acid hydrolysis and indicates that the more vigorous hydrolysis did not significantly alter the recovery of acids.

Table 1. Formation of acidic metabolites of tritiated P and DOC by rabbit liver slices

	% Formation of acids*			
	Progesterone (1.9 ng)		Deoxycorticosterone (3.5 ng)	
Wt. of tissue (mg)	With cofactors	Without cofactors**	With cofactors	Without cofactors
50	3.1	1.1	4.1	2.0
100	7.0	1.0	10.2	2.4
200	10.2	1.8	14.3	3.1
500	12.2	2.9	16.3	4.1

*Incubated 2 h, hydrolysed with Glusulase

**NADP (0.6 mM); glucose-6-phosphate (0.1 M); glucose-6-phosphate dehydrogenase (0.5 Units)

Table 2. Effect of incubation time on the formation of acidic metabolites by microsomal-supernatant liver fraction

	% Formation of metabolites*				
	Neutral		Acidic		
Incubation time (min)	^{3}H-P	^{3}H-DOC**	^{3}H-P	^{3}H-DOC	P value
15	58.8±1.9	60.1±5.7	11.1±0.8	15.2±5.4	<0.001
30	40.7±4.6	43.4±1.4	20.9±1.2	30.1±1.7	<0.001
60	36.4±0.7	37.2±2.2	32.2±2.2	36.9±3.3	<0.02
120	32.6±1.3	30.8±1.4	36.6±2.7	41.5±1.8	<0.01
Control+	72.0	66.2	1.7	3.0	

*Mean values of 6 experiments ±S.D.; 15% HCl hydrolysis

**2.3 ng P; 3.4 ng DOC

+Boiled microsomal-supernatant (10,000 g) fraction, presumably contains unchanged substrate.

Formation of acid steroid metabolites with liver subcellular fractions. ^{3}H-P and ^{3}H-DOC were incubated with increasing protein concentrations of M–S, microsomal, cytosol and mitochondrial fractions and the proportions of acids formed were determined at 120 min incubation. Figs. 1 and 2 illustrate the recovery of acids and indicate that maximal acid formation from both ^{3}H-P and ^{3}H-DOC is localised in the microsomal fraction. DOC again gave a greater yield of acids than P, and the cytosol only low levels of acids. Recombination of cytosol and microsomal fractions restored acid formation, presumably due to microsomal activity.

It was observed that similar proportions of neutral and acidic metabolites were obtained whether the subcellular fraction incubates were either directly extracted and solvent partitioned or hydrolyzed with hot acid and then solvent partitioned. This suggests that conjugate formation did not occur to a significant extent under the experimental conditions employed.

Methylation of acidic fractions. Evidence that the radiometabolites that partitioned into the sodium bicarbonate solution after acid hydrolysis were acidic compounds rather than highly polar water soluble metabolites was obtained by methylation and solvent partition. The neutral methyl ester fractions accounted for 74·5–94·2% of the ^{3}H-P acids and 70·7–86·0% of the ^{3}H-DOC acids.

Table 3. Effect of hydrolysis on the yield of acids from microsomal-supernatant incubations

	% Recovery of radioactivity incubated*			
	^{3}H-P**		^{3}H-DOC	
Hydrolysis	Neutrals	Acids	Neutrals	Acids
Glusulase	34.6±2.7	31.8±2.3	33.9±2.8	36.4±1.5
Hot acid	32.6±3.3	29.1±1.9	30.3±1.8	34.2±2.7
P value	0.3	0.2	0.1	0.2

*Mean of 4 experiments ±S.D.

**2.2 ng P; 3.5 ng DOC.

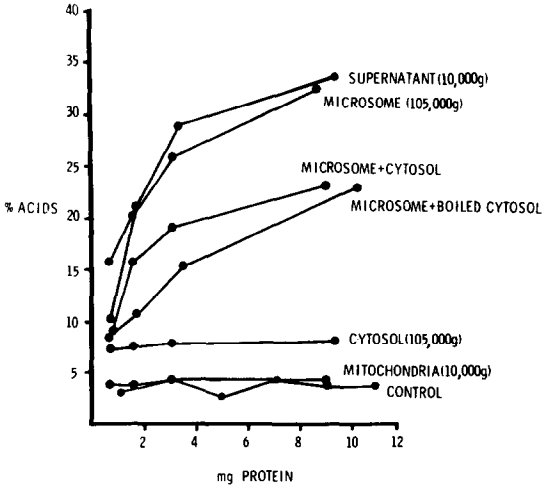

Fig. 1. Formation of acidic metabolites of [^{3}H]-progesterone with rabbit liver subcellular fractions.

DISCUSSION

Several species form steroid acids as metabolic products of corticosteroids, as either urinary metabolites [13–16] or as products isolated from the liver [11–21], placenta [22] or adrenal [23–27]. Recent reports suggest that the corticoid side chain undergoes an intramolecular rearrangement in the human, the most likely pathway being via the 20-hydroxy-21-aldehyde to the 20-hydroxy-21-oic acid [15, 16]. So far this reaction has not been studied *in vitro* with human liver, but studies on the sheep liver supernatant fraction indicated that an alternative pathway involving the oxidation of the 20-oxo-21-aldehyde to the 20-oxo-21-oic acid was operative [12].

The present study is the first reported *in vitro* formation of acidic metabolites of a C-21-deoxy steroid, progesterone, by rabbit liver. Methylation of the acids suggested that these compounds were carboxy compounds, but unlike the studies on the urinary metabolites [6] no evidence has been obtained so far that they are C-21-carboxylic acids. The microsomal fraction of rabbit liver was the most active subcellular fraction for the formation of acids from both progesterone and deoxycorticosterone. This contrasts with the localisation of the keto aldehyde dehydrogenase that converts 21-dehydrocorticoids to 20-oxo-21-oic acids in the supernatant fraction of sheep liver [12].

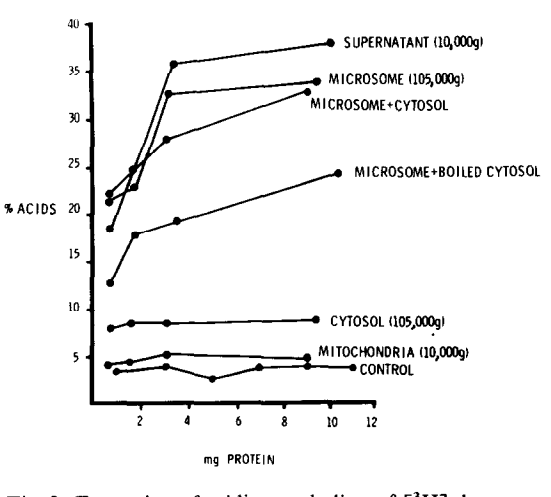

Fig. 2. Formation of acidic metabolites of [^{3}H]-deoxycorticosterone with rabbit liver subcellular fractions.

Rabbit liver homogenate is particularly active *in vitro* in the formation of glucuronide conjugates of progesterone metabolites [28]. Although the incubates were hydrolyzed in the present study, it was noted that direct solvent partition without hydrolysis gave comparable results indicating that conjugate formation was not extensive. A reason for this could be the known inhibition of glucuronide formation when propylene glycol is used as the steroid vehicle [29]. To date, the evidence from *in vivo* studies indicates that the acids are excreted predominantly as non-conjugated compounds in rabbit urine, with only small proportions occurring in the glucuronide fraction [6, 17]. The "water-soluble" metabolites of administered progesterone [30] and cortisone [31] observed in the alkali-washes of rabbit bile and urine may have consisted of acids, but were not associated with a particular conjugate fraction either.

Deoxycorticosterone formed acids *in vitro* at a faster rate than progesterone with the rabbit liver M–S fraction, though both steroids gave significant yields. This may indicate that while a C-21-hydroxyl function facilitates the formation of the acidic metabolites, its absence does not prevent their formation. If an intramolecular rearrangement similar to that proposed for cortisol [16], or direct oxidation of the 20-oxo-21-aldehyde, as occurs with sheep liver [12] is operative, a prerequisite would be the 21-hydroxylation of progesterone. In this context it is of interest that evidence for 21-hydroxylase activity in rabbit liver has been reported [32]. The acidic metabolites of progesterone isolated so far from rabbit urine have all been 6-oxygenated compounds [6, 8], and both 21- and 6β-hydroxylase activities are known to be localised in the microsomal fractions [33]. Since acid formation is also associated with this fraction, characterisation of the microsomal metabolites should allow the formulation of an acidic metabolic pathway.

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