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SHORT COMMUNICATION

LACK OF CORRELATION BETWEEN HEPATIC MICROSOMAL PROGESTERONE 21-HYDROXYLASE ACTIVITY AND THE EXCRETION OF ACIDIC METABOLITES IN RABBIT URINE

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Summary—Hepatic microsomes were prepared from liver sections removed from anesthetised New Zealand White rabbits. Two groups of rabbits, distinguished by different levels of progesterone 21-hydroxylase, were compared for *in vitro* and *in vivo* metabolism of [^{3}H]deoxycorticosterone/[^{14}C]-progesterone mixtures. *In vitro*, microsomes with low 21-hydroxylase activity gave significantly lower yields of pregnenoic acid than those with higher 21-hydroxylase activities. *In vivo*, these differences were not evident from an examination of the ^{3}H/^{14}C ratios of acidic urinary metabolites.

INTRODUCTION

Side-chain oxidation of progesterone by the New Zealand White (NZW) rabbit results in the excretion of 20-oxo-21-oic acids in the urine [1, 2]. The oxidative pathway has been demonstrated *in vitro* with liver microsomes to involve C-21-hydroxylation of progesterone to DOC*, followed by oxidation of the α-ketol to the 21-oic acid, identified by the accumulation of pregnenoic acid [3, 4]. The wide intraspecies variability in the level of progesterone 21-hydroxylase activity that has been documented with the NZW rabbit [5, 6] led us to determine the enzymatic level of individual rabbits with liver sections removed from anesthetised animals which subsequently served as their own controls [7]. Since variability in progesterone 21-hydroxylase activity might also be expected to influence steroid acid formation, the present investigation was undertaken to assess whether the excretion of acidic metabolites in the urine could provide an indirect index of 21-hydroxylase activity.

EXPERIMENTAL

Six NZW rabbits (3.5–4.75 kg) were anesthetised with Innovar-Vet (0.2 ml/kg b. wt given i.m.; M.T.C. Pharmaceuticals, Canada), together with Atropine sulfate (0.1 ml; Squibb, Canada Inc.). Liver sections were removed under sterile conditions and the mid line incision closed with surgical gut. After 4 weeks, the 5 surviving rabbits were housed in metabolic cages with access to water and Purina lab chow *ad lib*. Each rabbit was injected i.v. via the marginal ear with 1.0 ml of 0.9% saline containing [^{3}H]DOC (10 nmol; 17.88 μCi), [^{14}C]progesterone (10 nmol; 2.47 μCi) and 10% ethanol. The ^{3}H/^{14}C ratio was 7.24. Urines were collected for three consecutive 24 h periods and stored at −20°C until processed. Preparation of liver section microsomes, incubation conditions and partition of neutral and acidic metabolites was as previously described [3, 4, 6]. Microsomal protein (1.0 mg) was incubated with either [4-^{14}C]P (0.25 μCi) alone or with 4 nmol each of [^{3}H]DOC (0.25 μCi) and [^{14}C]P (0.1 μCi) for 30 min. Neutral and acidic metabolite fractions were run on Anasil-OF thin layer plates (250 μm; 5 × 20 cm, Analabs, U.S.A.) in chloroform–ethyl acetate (6:4, v/v) or ethyl acetate–formic acid (99:1, v/v) respectively. Radiometabolites were detected with a Radiochromatogram scanner (Packard, Model 7201) and compared to the location of u.v. absorbing internal standards. Corresponding areas of silica gel were scraped into test tubes, eluted with methanol and after evaporation counted in a PCS-Xylene (1:1, v/v) scintillation mixture (Amersham, Canada Ltd) in a Beckman liquid scintillation counter (LS-3801). Urinary metabolites were hydrolysed by either boiling aliquots (5 ml) with 15% HCl (v/v) for 10 min or by incubation with β-glucuronidase (500 U/ml; Glucurase, Sigma Chemical Co., U.S.A.) in sodium acetate buffer (0.1 M, pH 5.0) for 24 h. Neutral and acidic metabolites were extracted, partitioned and quantitated as previously described [1].

[1,2-^{3}H]Deoxycorticosterone (50 Ci/mmol) and [4-^{14}C]-progesterone (54.2 mCi/mmol) were obtained from New England Nuclear (Canada) Ltd, and non-labelled steroids from Steraloids Inc., U.S.A.

RESULTS AND DISCUSSION

Liver section microsomes prepared from anesthetised rabbits have been shown to possess similar enzymatic activities to those prepared from the total liver following sacrifice [7]. Table 1 gives comparative data on the microsomal yield of rabbits of different weight and sex and Fig. 1 the neutral [^{14}C]P metabolite spectrum obtained at 30 min incubation. Two groups of rabbit could be differentiated on the basis of their liver microsomal activities which were not influenced by age, sex, weight or microsomal yield. One group of rabbits, exemplified by nos 1–3, showed a relative deficiency of progesterone 21-hydroxylase activity identified by the absence of a radiometabolite peak corresponding to DOC in the radiochromatogram spectrum. A more mobile peak, identified as pregnanolone in a previous

Abbreviations: DOC, 21-hydroxy-4-pregnene-3,20-dione; P, progesterone, 4-pregnene-3,20-dione; 16α-OHP, 16α-hydroxy-4-pregnene-3,20-dione; 6β-OHP, 6β-hydroxy-4-pregnene-3,20-dione; 6β-OHDOC, 6β,21-dihydroxy-4-pregnene-3,20-dione; Pregnenoic acid, 4-pregnen-3,20-dion-21-oic acid.

Table 1. Preparation of liver section microsomes from anesthetised rabbits

Rabbit no.	Sex	Liver wt (kg)	Liver section (g·wet wt)	Microsomal protein (mg·g·wet wt^{-1} liver)
1	F	4.25	4.96	10.7
2	M	3.74	4.07	15.0
3	F	5.25	5.33	12.6
4	F	4.75	3.53	23.2
5	F	5.0	3.59	16.2
6	M	3.5	3.39	23.1

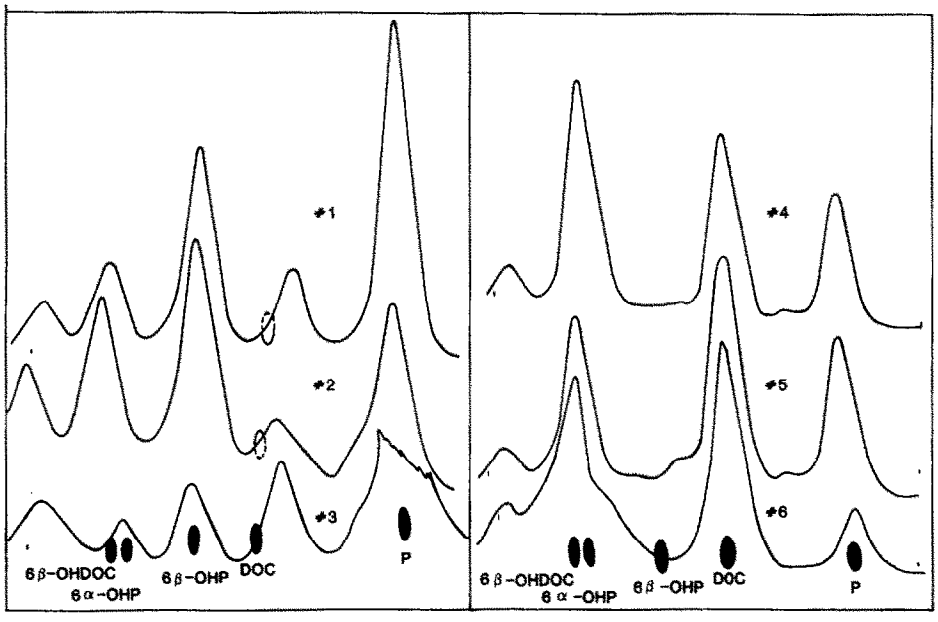

Fig. 1. Radiochromatogram scans of neutral progesterone metabolites separated by thin layer chromatography to show the designation of two groups of NZW rabbits based on the level of progesterone 21-hydroxylase activity.

study [8], was consistently present in this group and overlapped the DOC zone. Overall metabolism by this group of rabbits also tended to be reduced compared to the second group of rabbits, exemplified by nos 4–6, which gave liver microsomes with significantly higher levels of progesterone 21-hydroxylase activity. Rates of DOC formation by rabbits nos 4, 5 and 6 were 1.10, 0.95 and 1.60 nmol mg protein^{-1} min^{-1} respectively based on 5 min incubations with 0.1 mg microsomal protein. Under these conditions DOC formation by rabbits nos 1–3 could not be detected.

Table 2. Comparison of pregnenoic acid formation by liver section microsomes exhibiting different 21-hydroxylase activities

Rabbit no.	Pregnenoic acid ^{3}H	Pregnenoic acid ^{14}C	^{3}H/^{14}C Ratio
1	19.0	1.2	15.6
2	53.6	7.7	7.0
3	29.9	4.9	6.1
Mean ± SD	34.2 ± 17.7	4.5 ± 3.3	9.6 ± 5.2
4	83.9	49.3	1.7
5	54.2	51.8	1.05
6	50.1	48.4	1.04
Mean ± SD	62.7 ± 18.4	49.8 ± 1.8	1.26 ± 0.38

Results are expressed as pmols pregnenoic acid·mg protein^{-1}, 30 min^{-1} incubation derived from [^{3}H]DOC and [^{14}C]P respectively. Rabbits nos 1–3 exhibited low microsomal progesterone 21-hydroxylase activity.

Both groups of rabbits exhibited active 6β-hydroxylation and 6β-OHP accumulated with most microsomal incubates at 30 min, together with variable amounts of 6β-OHDOC.

The recognition of a wide variation of hepatic progesterone 21-hydroxylase activity in the outbred NZW rabbit, which has been suggested to fall into a bimodel distribution [5], allows the selection of rabbits having widely different activities. Since previous studies have shown that rabbit liver microsomes oxidise progesterone to pregnenoic acid via the 21-hydroxylation to DOC [3, 4], it was anticipated that the formation of pregnenoic acid would be influenced by the level of 21-hydroxylase activity. This was confirmed as shown in Table 2 which compares the metabolism of [^{3}H]DOC/[^{14}C]P mixtures by microsomes from the two groups of rabbits. Microsomes from rabbit nos 1–3 with low 21-hydroxylase activity gave higher ^{3}H/^{14}C pregnenoic acid ratios. It was also evident that α-ketol oxidase activity was lower in two out of three rabbits exhibiting low

Table 3. Excretion of radioactivity in 1st 24 h urine

Rabbit no.	Urine vol. (ml)	% of dose ^{3}H	% of dose ^{14}C	^{3}H/^{14}C
1	300	53.1	63.0	6.1
2	200	57.4	72.5	5.7
4	350	56.3	64.8	6.2
5	600	49.2	56.4	6.33
6	200	16.8	20.5	5.9

Table 4. $^3H/^{14}C$ Ratios in hydrolysed urine fractions

Rabbit no.	Neutral metabolites		Acid metabolites	
	HCl*	Glucuronidase†	HCl	Glucuronidase
1	8.0	6.4	6.0	8.0
2	4.7	8.8	6.0	5.7
4	12.9	9.3	5.9	6.2
5	7.5	12.4	6.0	6.1
6	9.8	14.6	5.0	5.5

*Boiled with 15% HCl for 10 min.
†Incubated with β-glucuronidase for 24 h.

microsomal 21-hydroxylase activity as shown by a reduced conversion of [^{3}H]DOC to [^{3}H]pregnenoic acid. Since the relative conversion of progesterone to pregnenoic acid proved an accurate index of microsomal progesterone 21-hydroxylase activity, it was of interest to determine whether the excretion of acidic progesterone metabolites in rabbit urine could serve as an indirect index of *in vivo* activity. Five of the original six rabbits that survived the liver biopsy were injected with the same doses of [^{3}H]DOC/[^{14}C]P. Table 3 shows that there was a comparable excretion of radioactivity in variable urine volumes during the first 24 h period. The decline in $^3H/^{14}C$ ratio from the original 7.24 was consistent with the known more fascile urinary secretion of progesterone metabolites in this species. The ratios of $^3H/^{14}C$ in the neutral and acidic metabolite fractions assessed after hydrolysis are given in Table 4. Both groups of rabbits gave similar $^3H/^{14}C$ acidic metabolite ratios which failed to reflect the different levels of 21-hydroxylase activity exhibited by liver microsomes *in vitro*. The significance of this observation remains to be assessed.

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