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CIRCADIAN RHYTHM OF HEPATIC STEROID METABOLIZING ENZYME ACTIVITIES IN THE RAT

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SUMMARY

The activity of various rat-liver microsomal steroid metabolizing enzymes was shown to follow a circadian rhythm. NADPH: 4-ene-3-oxosteroid-5α-reductase in female rats exhibited a maximum at 18.00 h and a minimum at 6.00 h. The same enzyme in male rats showed a minimum at 18.00 h (substrate testosterone) and 12.00 h (substrate corticosterone) and a maximum at 24.00 h. Variations in testosterone hydroxylation and NADPH-cytochrome c reductase activity were only detectable in male rats (maximum at 24.00 h and minimum at 12.00 h). NADH: 4-ene-3-oxosteroid-5α-reductase activity in male and female rats was highest at 6.00 h and lowest at 18.00 h.

INTRODUCTION

Several liver enzyme activities exhibit rhythmic changes over a 24 h period. Such rhythmic variations in enzyme activities were described for drug metabolizing enzymes [1, 2], cholesterol-7α-hydroxylase [3], hydroxymethylglutaryl-CoA-reductase [4], tyrosine transaminase [5], phenylalanine hydroxylase [6] and histone phosphorylase [7]. We have studied whether steroid metabolizing enzymes in rat liver microsomes (NADPH: 4-ene-3-oxosteroid-5α-reductase, NADH: 4-ene-3-oxosteroid-5α-reductase, testosterone hydroxylases and 3α- and 3β-hydroxysteroid dehydrogenases) exhibit circadian variations, too. Former studies on rhythmic variations were performed with male rats. In our experiments the enzyme activities were measured in male and female rats to detect sex differences.

EXPERIMENTAL

Animals. Male and female rats (Wistar) aged about 30 days and weighing 180–200 g were obtained from Zentralinstitut für Versuchstiere, Hannover, Germany. They were kept in a room lightened from 6.00 to 18.00 h for 14 days. At 6 h intervals, beginning at 6.00 h, four male and four female rats were killed, the livers were removed and microsomes were prepared immediately.

Enzyme assays. NADPH: 4-ene-3-oxosteroid-5α-reductase activity was measured in 3.0 ml reaction mixture containing 0.2 M Tris buffer (pH 6.25), 0.1 mM testosterone, 0.4 mM NADPH and 0.08–0.13 mg microsomal protein. After an incubation time of 20 min the formed products were extracted twice with diethylether and assayed as trimethylsilylethers by gas liquid chromatography [8]. The 5α-reduction of corticosterone was performed in 0.2 M potassium phosphate buffer (pH 7.0) (final volume 3.0 ml) with 0.1 mM corticosterone, 0.4 mM NADPH and 0.16–0.25 mg microsomal protein. The reaction products were determined as O-methoxime-trimethylsilylethers [9]. NADH: 4-ene-3-oxosteroid-5α-reductase activity in rat liver microsomes was determined as previously described [10]. Testosterone hydroxylation was carried out in 0.2 M potassium phosphate buffer (pH 7.0) with 0.4 mM NADPH, 0.1 mM testosterone (containing 0.5 μ Ci [^{3}H]-testosterone) and 0.16–0.25 mg microsomal protein. After the incubation (30 min, 37°C) steroid products were extracted by ether. The hydroxylation products of testosterone were separated from testosterone by thin-layer chromatography on silica gel HF. The thin-layer plate was developed twice in cyclohexane–ethyl acetate (60:40, V/V). The hydroxytestosterones were eluted from the silica gel by 2 × 2 ml methanol. After evaporation of the eluate the residue was transferred to a counting vial by means of 2 × 100 μl benzene. Ten millilitres of liquid scintillator KL 454 (Koch-Light Lab.) were added and the radioactivity was counted in a Liquid Scintillation Counter from Nuclear Chicago, Model Mark I. Cytochrome P-450 concentration was determined in a reduced CO-difference spectrum using an extinction coefficient of 91 cm^{-1}. mM^{-1} [11]. NADPH: 3α- and 3β-hydroxysteroid dehydrogenase activities were assayed with 5α-dihydrotestosterone as substrate by gas liquid chromatography of the formed products [12]. NADPH-cytochrome c reductase was measured spectrophotometrically [13]. The concentration of protein was determined by the Biuret reaction [14].

For statistical analysis Student's *t*-test was employed.

RESULTS

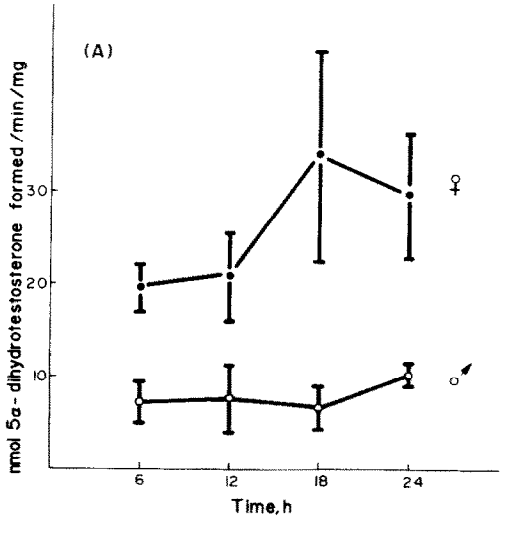
The NADPH: 4-ene-3-oxosteroid-5α-reductase (substrate testosterone) of female rats showed a maximum at 18.00 h and a minimum at 6.00 h. Microsomes of male rats had a specific activity of about 7 mU/mg between 6.00 and 18.00 h and a maximum at 24.00 h (10.31 ± 1.2 mU/mg) (Fig. 1A).

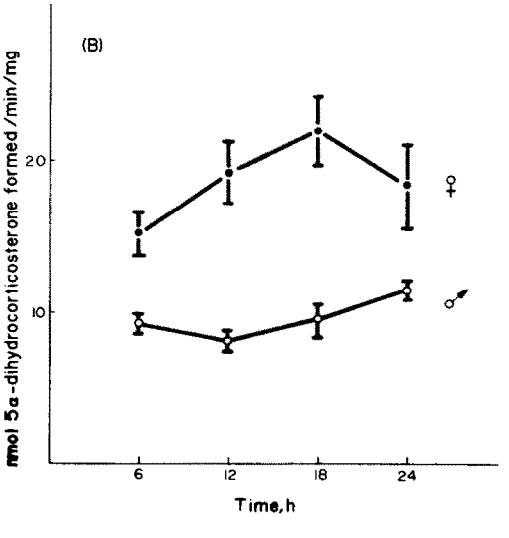
The corticosterone 5α-reduction with NADPH in the liver of female rats exhibited a maximal activity at 18.00 h and a minimum at 6.00 h. The activity in microsomes of male rats was highest at 24.00 h and lowest at 12.00 h (Fig. 1B).

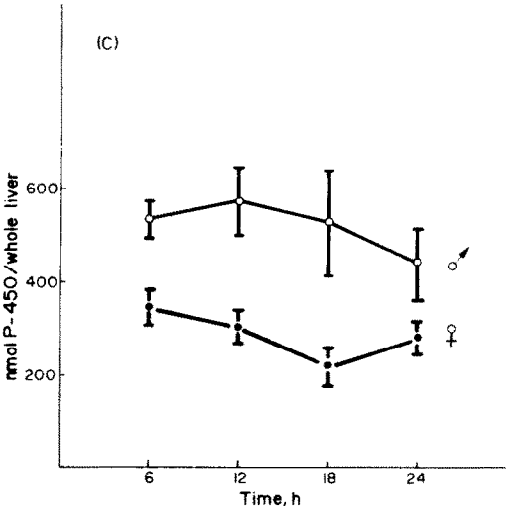
Another rhythm was noted in the NADH: 4-ene-3-oxosteroid-5α-reductase. The rhythmic pattern of this enzyme in male and female rats was characterized by a maximum at the start of the light period (6.00 h) and a minimum in activity at 18.00 h (Table 1).

In male rats an important pathway of testosterone metabolism is the hydroxylation which was found to be greater in male than in female rats. It was observed that the testosterone hydroxylation in male rats had a maximum at 24.00 h and a minimum at 12.00 h. The 24.00 h activity was twice as high as the 12.00 h activity (Fig. 1 D). No circadian variations of testosterone hydroxylation were found in the liver of female rats. When the testosterone hydroxylase activity in male rats was calculated for whole liver, a similar profile with maximum at 24.00 h (778 ± 173 nmol/min/liver) and a minimum at 12.00 h (491 ± 88 nmol/min/liver) was obtained.

Since NADPH-cytochrome c reductase is involved in the NADPH: 4-ene-3-oxosteroid-5α-reduction [15], NADH: 4-ene-3-oxosteroid-5α-reduction (16) and testosterone hydroxylation [17, 18] the circadian variations of this enzyme were studied. The maximum activity of NADPH-cytochrome c reductase in male







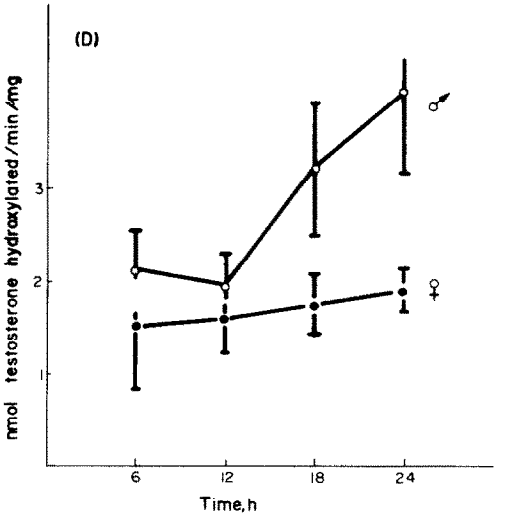


Fig. 1. Circadian variations in rat liver microsomal enzyme activities: (A) NADPH: 4-ene-3-oxosteroid-5α-reductase (substrate testosterone); (B) NADPH: 4-ene-3-oxosteroid-5α-reductase (substrate corticosterone); (C) Cytochrome P-450 content in whole liver; (D) Testosterone hydroxylation.

Table 1. Circadian variations of various microsomal steroid-metabolizing enzyme activities in rat liver

Enzyme	Male rats S.A. (nmol/min.mg)	Time (h)	Female rats S.A. (nmol/min.mg)	Time (h)
3α-hydroxysteroid dehydrogenase	9.69 ± 2.89	6.00	7.09 ± 0.59	18.00
P value	11.63 ± 1.53	24.00	5.42 ± 1.29	24.00
	†N.S.		<0.05	
3β-hydroxysteroid dehydrogenase	4.98 ± 0.87	18.00	1.09 ± 0.25	12.00
P value	5.48 ± 1.35	24.00	1.59 ± 0.32	18.00
	†N.S.		<0.025	
NADPH: 5α-reductase (testosterone)	6.82 ± 2.39	18.00	19.57 ± 2.48	6.00
P value	10.31 ± 1.20	24.00	34.13 ± 11.31	18.00
	<0.025		<0.025	
NADPH: 5α-reductase (corticosterone)	8.19 ± 0.63	12.00	15.24 ± 1.43	6.00
P value	11.59 ± 0.60	24.00	22.00 ± 2.45	18.00
	<0.0005		<0.0025	
NADH: 5α-reductase* (testosterone)	11.07 ± 3.17	6.00	31.99 ± 15.95	6.00
P value	8.28 ± 3.69	18.00	21.60 ± 7.95	18.00
	<0.05		<0.05	
NADPH: cytochrome c-reductase	144.60 ± 29.30	12.00	157.20 ± 22.60	6.00
P value	201.60 ± 25.20	24.00	135.90 ± 23.40	18.00
	<0.025		†N.S.	
Testosterone hydroxylation	1.95 ± 0.32	12.00	1.51 ± 0.72	6.00
P value	4.95 ± 0.91	24.00	1.90 ± 0.20	24.00
	<0.0025		†N.S.	
Cyt. P-450 (nmol/mg)	2.22 ± 0.29	6.00	1.82 ± 0.12	12.00
P value	2.38 ± 0.53	18.00	1.37 ± 0.14	18.00
	†N.S.		<0.0025	
Cyt. P-450 (nmol/whole liver)	571.13 ± 78.62	12.00	344.78 ± 41.02	6.00
P value	442.13 ± 79.67	24.00	221.63 ± 37.22	18.00
	<0.05		<0.0025	

Only maximal and minimal activities ± standard deviation during the 24 h period are listed above. Four animals per group were used.

* 10 animals per group.

† Not significantly different ($P > 0.05$).

rat liver microsomes was found at 24.00 h, the minimum was at 12.00 h (Table 1).

This is in agreement with the results reported by Tredger *et al.*[1]. In female rats no significant variations of NADPH-cytochrome c reductase activity were observed.

The cytochrome P-450 content was measured in liver microsomes of male and female rats. When the results are expressed in nmol/mg protein, there was no clear maximum in male rats, however, we could observe a maximum at 12.00 h and a minimum at 18.00 h in female rats (Table 1). The cytochrome P-450 content of the whole liver showed a peak at 12.00 h and a trough at 24.00 h in male rats. Female rats had a maximum at 6.00 h and a minimum at 18.00 h (Fig. 1C).

No significant circadian variations were found for NADPH-3α- and 3β-hydroxysteroid dehydrogenase in male rats. In female rats NADPH-3α-hydroxysteroid dehydrogenase was highest at 18.00 h and lowest at 24.00 h. NADPH-3β-hydroxysteroid dehydrogenase activity exhibited a maximum at 18.00 h and a minimum at 12.00 h. Minimum and maximum activities were significantly different (Table 1).

DISCUSSION

The steroid metabolizing enzymes in rat liver microsomes showed variations in activity within 24 h. It seems that there are three types of rhythms:

(1) Maximum at 18.00 h and minimum at 6.00 h was found in NADPH: 4-ene-4-oxosteroid-5α-reductase activity with testosterone and corticosterone as substrate in female rats.

(2) Maximum at 24.00 h and minimum at 12.00 h was observed in NADPH: 4-ene-3-oxosteroid-5α-reduction of corticosterone in male rats. The maximum of NADPH: 4-ene-3-oxosteroid-5α-reduction of testosterone in male rats was likewise at 24.00 h, however, minimum activity was found at 18.00 h. The maximum is shifted for 6 h compared with female rats. It is possible that these types of rhythms are influenced by the adrenal cortex or the plasma corticosterone concentration which is increasing from the morning to the evening. This was supposed by Radzialowski and Bousquet[2] for drug metabolizing enzymes in rat liver microsomes and by van Cantfort[19] for cholesterol-7α-hydroxylase. The same type of rhythm was found in testosterone hydroxylation and NADPH-cytochrome c reductase activity in male rats. No significant variations of these enzyme activities were found in liver microsomes of female rats. A similar type of rhythm was described for drug metabolizing enzymes in microsomes of male rats with a maximum at 24.00 h and a minimum at

14.00 h [1]. These authors found the highest activity of NADPH-cytochrome c reductase at 24.00 h and a minimum in activity at 14.00 h, which is in agreement with our results. A similar rhythm with maximum activity around 22.00 h was found for cholesterol-7α-hydroxylase in male rat liver [3].

(3) Another rhythm exhibits the NADH: 4-ene-3-oxosteroid-5α-reductase activity in male and female rats with a maximum at 6.00 h and a minimum at 18.00 h. The different behaviour of NADH: 4-ene-3-oxosteroid-5α-reductase and NADPH: 4-ene-3-oxosteroid-5α-reductase demonstrates that they are different enzyme systems. Serum testosterone concentration was found to be highest in the morning [20, 21]. Therefore it might be possible that testosterone plays a regulatory role in the variations of NADH: 4-ene-3-oxosteroid-5α-reductase activity. *In vivo* testosterone is possibly the preferred substrate for NADH: 4-ene-3-oxosteroid-5α-reductase, since the activity of the enzyme is five times as high for testosterone than for corticosterone [22].

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