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DIURNAL VARIATION AND MELATONIN INDUCTION OF HEPATIC MOLYBDENUM HYDROXYLASE ACTIVITY IN THE GUINEA-PIG

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Abstract—The activities of the xenobiotic metabolizing enzymes, aldehyde oxidase and xanthine oxidase, were determined in partially purified fractions of adult guinea-pig liver at given times in the day or night. A marked circadian variation in aldehyde oxidase activity was observed with several substrates (phthalazine, phenanthridine, *N*-phenylquinolinium and 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone). The main peak occurred at 0300 hr with minimum activity from 1200 to 1800 hr, the differences between rhythmic extremes being statistically significant ($P < 0.005$). Xanthine oxidase activity also exhibited a daily rhythm but with a lower amplitude. Guinea-pig serum melatonin showed a synchronous circadian fluctuation with peak values at 0300 hr falling throughout the day to a minimum at 1800 hr. Exogenously administered melatonin caused a significant increase in aldehyde oxidase activity at 0900 and 1200 hr and in xanthine oxidase activity at 0900 hr. It was concluded that melatonin concentrations may be related to the circadian variation in liver molybdenum hydroxylase activity.

The cytosolic enzymes, aldehyde oxidase (EC 1.2.3.1) and xanthine oxidase (EC 1.2.3.2), are important in the biotransformation of numerous *N*-heterocycles and aldehydes ([1] and references contained therein). These molybdenum-containing enzymes are generally involved in nucleophilic oxidation although aldehyde oxidase in particular has been shown to catalyse many reduction reactions [2]. Xanthine oxidase is responsible for the oxidation of hypoxanthine to xanthine and uric acid [3] in the final stage of endogenous purine catabolism whereas aldehyde oxidase is thought to contribute to the oxidation of physiological substrates such as N^1-methylnicotinamide, pyridoxal and vitamin A aldehydes [4, 5]. However, both enzymes are also involved in the metabolism of a number of drugs including allopurinol, quinine and methotrexate [2].

A number of workers have reported diurnal rhythms, measured *in vitro*, for hepatic and extrahepatic drug-metabolizing enzymes; thus temporal variations have been found for the microsomal monooxygenases [6–14] and several transferase enzymes such as glutathione transferase, UDP-glucuronyltransferase and sulphotransferase [15, 16]. In most cases, maximum enzyme activity has been observed in the dark period with a corresponding minimum sometime during the light period [6–16]. Since light interacts with both the hypothalamic and pineal neuroendocrine systems, circulating corticosterone and plasma melatonin (MT) levels exhibit daily variations, respectively [17]. Some evidence suggests a reciprocal relationship between corticosterone concentrations and the daily rhythm in oxidative drug metabolism [7–11]. MT secretion, however, occurs in the dark phase of all species studied at a time of maximal metabolic enzyme activity [18].

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The aim of the present work was to investigate whether molybdenum hydroxylases exhibit circadian variations in activity. Studies were carried out using guinea-pigs because liver from this species has a reasonably high content of both molybdenum hydroxylases, whereas in rat and rabbit liver one enzyme predominates, i.e. xanthine oxidase and aldehyde oxidase, respectively [19, 20]. In addition, the relationship between MT and guinea-pig liver enzymes was investigated to determine whether MT, exogenously administered, could increase liver molybdenum hydroxylase activity.

MATERIALS AND METHODS

Preparation of enzyme for circadian studies. Adult male Dunkin–Hartley guinea pigs, aged 6 to 8 weeks and weighing 500 g were used throughout the study. They were housed in groups of three and allowed food and water *ad lib*. All animals were maintained in a regime of strictly controlled temperature (18 ± 1°), humidity (50–55%) and a lighting cycle of 0700–2100 hr light: 2100–0700 hr dark for at least 3 weeks prior to experimentation. Guinea-pigs were killed by cervical dislocation; blood was collected from the jugular vein into polypropylene centrifuge tubes, centrifuged and assayed for melatonin. The liver was rapidly excised free of the gall bladder and placed in ice-cold 0.154 M KCl. Each liver was blotted dry, weighed and homogenized in 2 to 3 vol. of ice-cold 0.154 M KCl in an Atomix liquidiser at high speed for 2 min. Partially purified enzyme was prepared from the homogenate as described previously for rabbit liver molybdenum hydroxylases [21]. In the present study, the precipitate obtained by ammonium sulphate saturation was dissolved in approximately 5 ml 0.1 mM EDTA, assayed immediately and the remainder stored as pellets in liquid N_2.

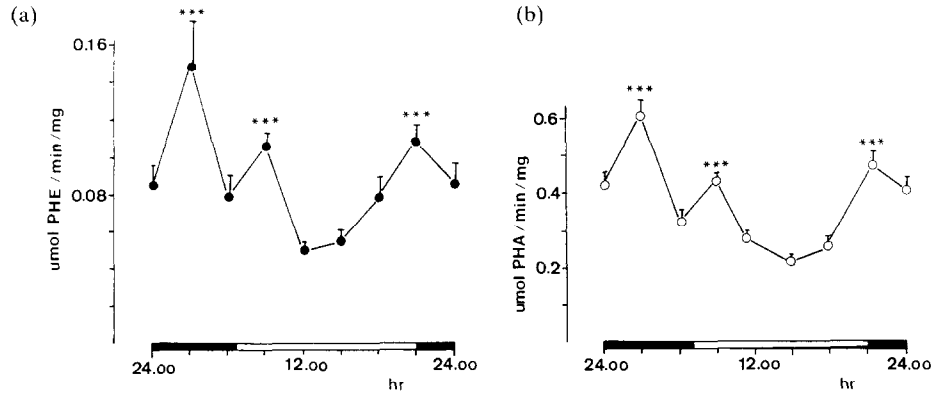


Fig. 1. Circadian variation in hepatic aldehyde oxidase activity. Enzyme activity was measured at 37° and expressed as μmol substrate (phenanthridine; PHE, phthalazine; PHA) consumed/min/mg protein. Each point represents the mean ± SE of five or six animals. *** Differences between maxima and minimum were highly significant ($P < 0.005$) using a two-tailed Students *t*-test.

Enzyme assay. Sources for the chemicals are given in the references quoted. Aldehyde oxidase activity was determined as described previously by Johnson *et al.* [22], for phthalazine, 3-methylisoquinoline and phenanthridine. Oxidation of 1 mM *N*-phenylquinolinium perchlorate [23] was monitored at 420 nm under the same conditions as phthalazine and dehydrogenation of 1 mM 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone [24] was measured by following the increase in absorbance at 310 nm corresponding to product formation. Xanthine oxidase activity was determined using 0.05 mM xanthine, either at 420 nm by following potassium ferricyanide reduction or at 295 nm as previously reported [19]. Protein concentration was estimated by the method of Bradford [25].

Measurement of locomotor activity. Locomotor activity was assessed using guinea-pigs, housed in groups of two or three in plastic cages with stainless steel wire tops under the conditions described above, which were placed on Automex activity meters. Readings were recorded automatically every 30 min over a period of 72 hr and expressed as a mean count for the end of each hour.

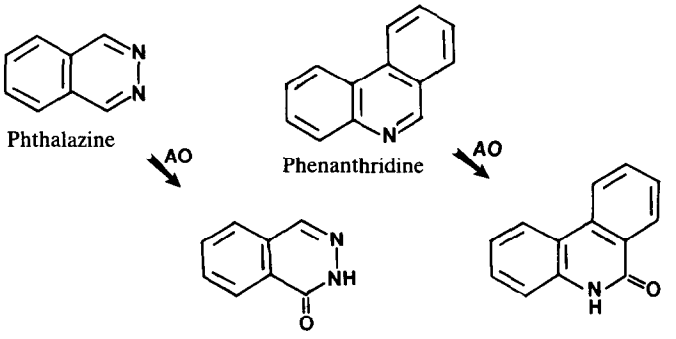
Preparation of enzyme for induction studies. Groups of guinea pigs (N = 6), housed as previously described, were administered MT in the drinking water (10 mg/kg/day) *ad lib*. This route of administration was chosen because MT in the serum has a very short half-life (~20 min) [26]. Control groups (N = 6) received water *ad lib*. After 5 days, the animals were killed at 0900 or 1200 hr and serum MT was determined as described earlier. Molybdenum hydroxylase activity was determined in partially purified fractions prepared, as above, from the 100,000 *g* supernatant.

Radioimmunoassay. *N*-[1-aminoethyl-2-^{3}H]-acetyl-5-methoxytryptamine (3HMT) 38.6 Ci/m mol was purchased from New England (Dreieich, F.R.G.) and MT was obtained from Sigma Chemical Co. (Poole, U.K.). Serum MT concentrations were measured using the radioimmunoassay reported by Bradbury *et al.* [27]. The detection limit of the assay was between 5 and 10 pg/ml of serum and the intra-assay coefficients of variation were 2.9, 7.2 and 15.7% at 40, 80 and 120 pg, respectively. The inter-assay coefficients of variation were 4.7 and 7.7% at 40 and 80 pg and recovery of ^{3}H–MT was 73, 86 and 91% at 20, 40 and 80 pg, respectively.

RESULTS AND DISCUSSION

The activity of both guinea-pig molybdenum hydroxylases, assayed at 4-hourly intervals, was found to vary markedly over a 24 hr period under controlled environmental lighting. Figure 1 shows the temporal variation in aldehyde oxidase activity monitored directly at 310 nm due to the formation of phenathridone from phenanthridine utilising molecular oxygen as an electron acceptor (Fig. 1a) and indirectly by following the reduction of an artificial electron acceptor, potassium ferricyanide, at 420 nm (Fig. 1b). Similar results were obtained in each case. The enzyme exhibited maximum oxidative activity in the middle of the dark period around 0300 hr and intermediate peak activity at 0900 and 2130 hr whereas the rate of oxidation decreased by about 60% during the day to give a minimum between 1200 and 1500 hr. The differences between the minimum and any of the peak maxima were highly significant ($P < 0.005$) in each case. The drop in activity observed in the oxidation of phthalazine and phenanthridine at 0600 hr was not always apparent when other substrates such as 3-methylisoquinoline were employed in the assay (data not shown). However, the decrease occurring after the onset of the dark period between 2130 and 2400 hr was present irrespective of the substrate used.

Phthalazine and phenanthridine are typical uncharged substrates of aldehyde oxidase; thus the oxidation products are both cyclic lactams substituted *adjacent* to a heterocyclic nitrogen atom. On the other hand, compounds such as cinnoline (1,2-diazanaphthalene) and *N*-heterocyclic cations undergo reaction at an electron-deficient carbon elsewhere in the heterocyclic ring [19, 23]. Guinea-pig liver aldehyde oxidase catalyses the oxidation of *N*-phenylquinolinium perchlorate predominantly to the



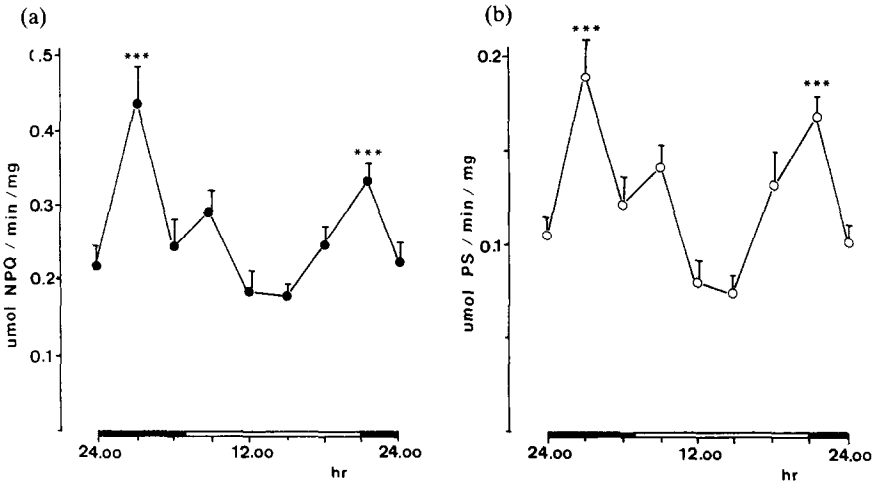
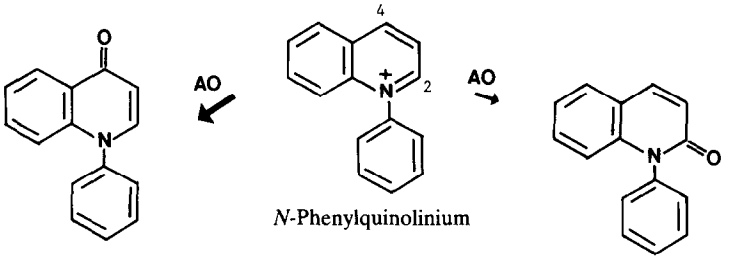
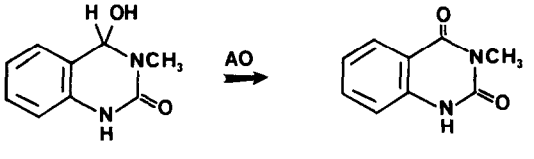


Fig. 2. Circadian variation in the activity of hepatic aldehyde oxidase towards charged substrates. Enzyme activity is expressed as μmol *N*-phenylquinolinium (NPQ) oxidized/min/mg protein and μmol 3,4-dihydro-4-hydroxy-3-methylquinazolinone (PS) dehydrogenated/min/mg protein (N = 5–6). *** $P < 0.005$.



N-Phenylquinolinium

4-quinolone although the isomeric 2-quinolone accounts for approximately 5–10% total product formed [23]. Figure 2(a) shows the circadian variation in the *N*-phenylquinolinium perchlorate oxidative activity of aldehyde oxidase. Similar rhythmic changes to those observed with uncharged substrates were observed. This was also the case when enzymic activity towards the pseudobase, 3,4-dihydro-4-hydroxy-3-methyl-2-quinazolinone, was monitored (Fig. 2b). This compound undergoes a novel reaction with aldehyde oxidase which apparently involves dehydrogenation and not nucleophilic oxidative attack [24].



3,4-dihydro-4-hydroxy-
3-methyl-quinazolinone

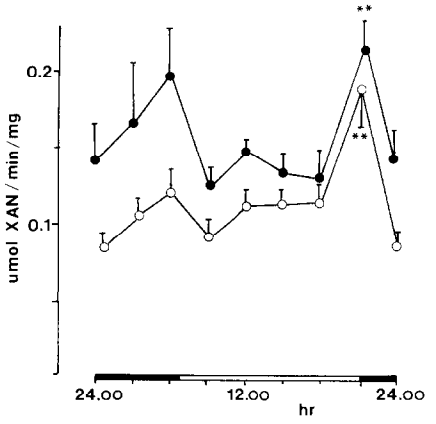


Fig. 3. Circadian variation in hepatic xanthine oxidase activity. Enzyme activity was measured at 37° using oxygen (●) as electron acceptor or in the presence of 1 mM potassium ferricyanide (○). Enzyme activity is expressed as μmol xanthine (XAN) oxidised/min/mg protein (N = 5–6). ** $P < 0.005$.

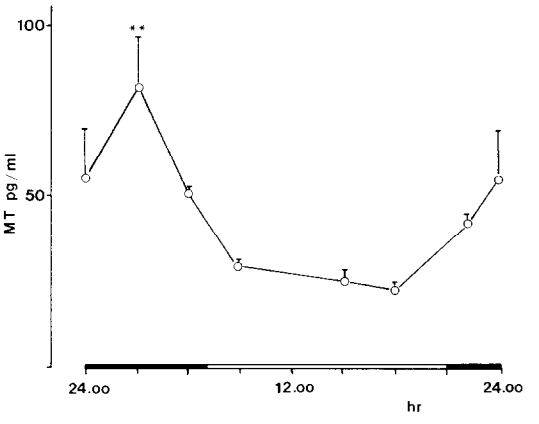


Fig. 4. Circadian variation in guinea-pig serum melatonin. Results are expressed as pg melatonin (MT) per ml of serum (N = 4–6). *** $P < 0.006$.

In contrast to the highly significant changes demonstrated in aldehyde oxidase activity the variation in xanthine oxidase activity was less marked (Fig. 3). Peak values were observed at 2130 hr, again followed by a significant drop ($P < 0.005$) at 2400 hr when xanthine oxidation was monitored directly using oxygen as the electron acceptor (●) or indirectly in the presence of ferricyanide (○). Enzyme activity was monitored with two electron acceptors because of the interconversion between xanthine oxidase and xanthine dehydrogenase which can occur during the purification procedure. *In vivo* the molybdenum hydroxylase responsible for xanthine oxidation is thought to exist mainly in a dehydrogenase form i.e. it is reoxidised via NAD^+ rather than O_2 [28]. This form can undergo facile conversion to an oxidase form during purification or on storage [29]. The enzyme from rat liver is almost all converted to the oxidase during heat treatment but the interconversion has not been studied with guinea-pig enzyme. Nevertheless, the values for the specific activities of xanthine oxidation are different depending on which electron acceptor is employed in the assay. The fact that O_2 reacts only slowly with xanthine dehydrogenase whilst ferricyanide is rapidly reduced by either form indicates that both types of the enzyme may be present. At the moment it is not clear which method best reflects enzyme activity *in vivo*. However, any variation in the activity of either form will lead to changes in endogenous purine catabolism and hence, serum uric acid levels. There are no reports of similar conversions occurring with aldehyde oxidase.

The relative timing of the peak and nadir of aldehyde oxidase activity within the light–dark cycle is similar to that previously reported for other drug metabolising enzymes [6–16]. Several workers have found that the circadian rhythm in hepatic hexobarbital oxidase and *O*-demethylase is dependent on environmental lighting and exposure of rodents to either continuous illumination or darkness abolished these rhythms [6, 8]. In addition, reversal of the lighting cycle also appeared to invert the rhythm in hexobarbital metabolism [6]. Nair and Casper [8] proposed that light may act as an inhibitory stimulus of microsomal oxidative drug-metabolising enzymes although the mechanism is not yet established. However, enzyme activity may also be affected by environmental factors other than the light–dark schedule. Thus microsomal *N*-demethylase cycles are influenced by the dietary protein level [14]. In the present study, the spontaneous locomotor activity of the guinea-pig showed a daily fluctuation which mirrored the rhythm observed in enzyme activity. Two peaks were seen, around 0300–0500 hr and a very sharp maximum at 2200 hr. Minima at 2400 and 1500 hr also characterise the profile. The changes between the rhythmic extremes were significantly different from the prior and subsequent extremes in activity. This diurnal rhythm may be due, in part, to actual consumption of food as the animals fed mainly during the dark period. Fasting could also have an effect on these rhythms, but conflicting reports have appeared such that the rhythm in glucuronyl transferase enzymes is abolished by fasting whereas microsomal oxidative activity does not appear to be altered [7, 11, 16].

Figure 4 demonstrates that guinea-pig serum melatonin levels undergo circadian fluctuation with peak values at 0300 hr and falling to a minimum during the light period. The ratio of dark:light levels of melatonin was approximately 4:1 and differences between the rhythmic extremes were significant ($P < 0.006$). A daily fluctuation in serum MT in experimental animals is well documented [18, 30]. However, variation in guinea-pig serum MT has not hitherto been previously reported although the data presented here is in agreement with that for all species so far studied. This circadian rhythm closely resembles that observed in molybdenum hydroxylase activity and is similar to variations in microsomal oxidative activity [11].

Exogenous administration of MT for 5 days caused a significant increase in the activity of hepatic aldehyde oxidase at 1200 hr (Table 1) using phthalazine,

Table 1. Effect of melatonin administration on guinea-pig liver molybdenum hydroxylases

	Specific activity at 37° and pH 7 (μmol/min/mg protein)			
	0900 hr		1200 hr	
Substrate	Control	MT-treated	Control	MT-treated
3-Methylisoquinoline	0.023 ± 0.002	0.0311 ± 0.003*	0.0159 ± 0.003	0.035 ± 0.003‡
Phthalazine	0.592 ± 0.04	1.33 ± 0.23*	0.389 ± 0.08	1.0 ± 0.09‡
Phenanthridine	0.068 ± 0.01	0.183 ± 0.02‡	0.076 ± 0.013	0.176 ± 0.02†
Xanthine	0.027 ± 0.005	0.049 ± 0.006*	N.D.	N.D.

The values are given as means ± SE (N = 6).
* Each activity following MT-treatment was significantly different from its corresponding control to at least $P < 0.05$ using a two-tailed Students *t*-test.
† $P < 0.004$.
‡ $P < 0.002$.

phenanthridine and 3-methylisoquinoline as substrates. Serum levels of MT in treated animals were approximately 500 times those of control guinea-pigs. Although the maximal oxidation rate for phthalazine was greater for aldehyde oxidase from MT-treated animals the Michealis–Menten constant was not significantly different from control values. Xanthine oxidase activity was unchanged at 1200 hr although at 0900 hr, both aldehyde and xanthine oxidase were increased in activity. In the same animals at 1200 hr the microsomal hydroxylation of aniline was increased although the microsomal *O*-demethylation of 4-nitroanisole did not appear to differ from control animals (results not shown).

An apparent relationship is indicated between serum MT and the activity of these drug-metabolizing enzymes particularly aldehyde oxidase, however the fact that pharmacological doses of MT increase their activity does not necessarily infer a causal relationship. Circadian variations in plasma corticosterone have an effect on basal rates of microsomal drug metabolism, but there is not a general dependence of these cycles on circulating corticosteroids [13, 14]. It is possible that melatonin could exert its effect by altering the food intake of the guinea-pigs. However, chronic administration of MT to mice causes only a slight augmentation of food intake [31] and there is no evidence to suggest that aldehyde oxidase levels would be affected if such an increase was to occur. In addition, although a low protein diet (~5%) results in the formation of inactive xanthine oxidase and hence decreases enzyme activity, an increase in dietary protein from 20 to 50% has *no* effect on xanthine oxidase activity [32].

Further investigations will establish whether aldehyde oxidase and P-450 hydroxylase are dependent on the variable secretions of the pineal hormone.

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