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The Circadian Variation of the Activity of Hepatic Molybdenum Hydroxylases in the Female Syrian Hamster

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ABSTRACT. The activities of molybdenum hydroxylases, aldehyde oxidase and xanthine oxidase, were determined eight times daily (at equal intervals) in partially purified preparations of liver of adult female hamsters. A marked diurnal variation of aldehyde oxidase activity was observed with the substrates used (phthalazine, 3-methylisoquinoline, phenanthridine). The maximum enzyme activity occurred at 0600 hr, whereas the minimum activity occurred at 0300. 0900 and 1800 hr. The differences between the maximum and minimum enzyme activities were highly significant ($P< 0.005$). Xanthine oxidase also showed circadian variation when xanthine was used as substrate. The maximum activity of xanthine oxidase occurred at 1500 hr whereas the minimum occurred at 0900 hr. The difference between the two extremes was statistically significant ($P<0.01$). These results indicate that hamster liver aldehyde oxidase and xanthine oxidase exhibit circadian variations in activity.

Microsomal monoxygenases or mixed function oxidases are involved in the biotransformation of a wide range of drugs and foreign compounds (Testa *et al.* 1981, Nakasa *et al.* 1993; and Cnubben *et al.* (1995). However, molybdenum hydroxylases, aldehyde oxidase (E.C.1.2.3.1) and xanthine oxidase (E.C. 1.2.3.2) present in the cytosol can also contribute to this process (Beedham, 1985; Beedham *et al.* 1987a; Beedham *et al.* 1992, Beedham *et al.* 1995; and Rashidi *et al.* 1997).

The activity of these enzymes has been found to have temporal variations in several species. Three early studies reported the presence of daily rhythmic variations in hepatic male rat microsomal enzymes (Radzialowski and Bousquet, 1968; Nair and Casper, 1969; and Jori *et al.* 1971). Moreover, the activity of microsomal enzymes exhibited circadian rhythms in drug metabolism in mice (Holcslaw, *et al.* 1975). Lake *et al.* (1976) observed a diurnal rhythm of microsomal enzymes in the golden hamster. Under a lighting cycle of 0630 to 1830 hr light, maximum activities were found between 0400 hr and 0800 hr

whereas minimum enzyme activities were between 1600 hr and 1800 hr. Furthermore, another group of microsomal enzymes, ethylmorphine N-demethylase, aniline hydroxylase and arylhydrocarbon hydroxylase, showed circadian rhythms in the male Syrian hamster (Birt and Hines, 1982). Molybdenum hydroxylases, which are involved in drug and xenobiotic metabolism, were demonstrated to show circadian variations in male guinea pigs (Beedham *et al.* 1989).

Aldehyde oxidase and xanthine oxidase are broadly distributed throughout the animal kingdom in primitive species like the sea anemone, crustaceans, molluscs, insects, birds, reptiles and majority of mammals (Andres, 1976; Hayden and Duke, 1975; Krenitsky *et al.* 1974; Wurzinger and Hartenstein, 1974; Al- Tayib, 2000 and Beedham, 1985). There are a few reports on multiple molybdenum hydroxylase iozymes. Holmes (1978) separated two aldehyde oxidase isozymes by electrophoresis from many mouse tissues and found them to be differentially distributed. In addition, Ohkubo *et al* (1983) separted two isozymes of aldehyde oxidase (N^1- methylnicotinamide oxidase) with different substrate specificity from rat liver by ion-exchange chromatography. Furthermore, Beedham *et al.* (1995) found different aldehyde oxidase isozymes in the livers of humans, guinea pigs, rabbits and baboons. The activity of guinea pig and human enzymes was similar. On the other hand, studies with xanthine oxidase indicated the presence of only a single form of the enzyme in a number of species (Bruder *et al.* 1984; and Seely *et al.* 1984; and Duley *et al.* 1985).

The present study was carried out using female hamsters because the liver of this species contains reasonable levels of aldehyde oxidase and xanthine oxidase, whereas in rabbit and rat liver one enzyme predominates respectively (Krenitsky *et al.* 1974, and Stubley *et al.* 1979).

The aim of this work was to study whether the phenomenon of daily variations in the activity of aldehyde oxidase and xanthine oxidase may take place in the hamster, since the circadian rhythm of both enzymes has not been investigated in this species.

Materials and Methods

Reference Compounds

Both phthalazine and phnanthridine were purchased from Aldrich Chemical Company. 3-Methylisoquinoline was obtained from ICN Pharmaceuticals Inc. Xanthine was supplied by Sigma Chemical Company.

Animals

Due to lack of enough male hamsters in the animal house, female hamsters were used in this investigation. Adult female Syrian hamsters weighing 40-45 g were obtained from King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. Hamsters were maintained on a standard laboratory diet (Oxoid, modified 41B) fed *ad libitum* with free access to water. All animals were housed in groups of 6, and maintained in a regime of a strictly controlled lighting cycle of 0600-1800 hr light and 1800-0600 hr dark, with a temperature of ($17\pm1^{o}C$) for 2 weeks prior to experimentation. They were killed by cervical dislocation, and their livers were rapidly collected free of the gall bladder, directly frozen in liquid nitrogen, and kept in a deep-freeze at $-80^{o}C$ for 2 weeks.

Preparation of Hepatic Enzyme

Aldehyde oxidase was partially purified according to the procedure reported by Johnson *et al.* (1987) with some modifications. Livers were weighed, finely chopped and homogenized in 3 volumes of ice-cold isotonic KCl (0.154 M) containing 0.1 mM EDTA for 30 sec with a polytron homogenizer. The resulting suspension was heated on a water bath at $50\text{-}55^{o}C$ for 15 min, immediately cooled to $10^{o}C$, then centrifuged at 20,000xg for 25 min at $4^{o}C$. Sufficient solid ammonium sulfate was added to the filtered supernatant to give 50% saturation with stirring for 15 min at $4^{o}C$ followed by centrifugation at 3000 xg for 15 min. The precipitate was washed with distilled water, and dissolved in 4 ml of 0.1 mM EDTA. The hepatic partially purified enzyme was stored in a deep-freeze at $-80^{o}C$ until used for spectrophotometric analyses.

Enzyme Assay

As a result of multiple forms of molybdenum hydroxylases, enzyme activity is monitored with different substrates. The activity of partially purified aldehyde oxidase was estimated in 67 mM phosphate buffer pH7 at 37°C using the method described previously by Johnson *et al.* (1984) with 3-methylisoquinoline (1mM) and phthalazine (1mM) at 420 nm, while the oxidation rate of phenanthridine was evaluated at 322 nm. Xanthine oxidase activity was determined with xanthine (50 μM) at 295 nm. Protein concentration was estimated by biuret method using bovine serum albumin as standard.

Results and Discussion

The interaction of aldehyde oxidase with nitrogen containing compounds has been found to vary widely from one species to another (Taylor *et al.* 1984; Kaye *et al.* 1984; Kaye *et al.* 1985; and Beedham *et al.* 1987a). The present study investigated the activity of hamster aldehyde oxidase and xanthine oxidase at 3-hourly intervals during a 24 hr period under a controlled lighting cycle. Figures 1 and 2 show the circadian variation of aldehyde oxidase activity with phthalazine and 3-methylisoqunioline indirectly at 420 nm by following the reduction of potassium ferricyanide as an electron acceptor. Both substrates had similar results with a maximum activity at the beginning of the light phase (0600 hr), and an intermediate peak at 2100 hr whereas the minimum activity was obtained at 0300, 0900 and 1800 hr. The differences between rhythmic extremes was statistically significant ($P < 0.005$). The enzyme activity was also monitored directly with phenanthridine using oxygen as an electron acceptor at 322 nm. Figure 3 shows a maximum peak at 0600 hr, and another middle peak at 2100hr. The minimum enzyme activity with phenanthridine occurred at three different times (0300, 0900 and 1800 hr). The differences between maixmum and any of the peak minima were significant ($P<0.01$). The presence of isozymes of aldehyde oxidase in mouse and rat had been reported by Holmes (1978) and Ohkubo *et al.* (1983). However, it can be observed from the above data that all the three substrates have the same time of maximum and minimum activity. This result shows that these compounds may work as substrates for the same form of aldehyde oxidase.

Oxygen as an electron acceptor was again used for the measurement of xanthine oxidase activity with xanthine at 295 nm. The maximum daily activity of xanthine oxidase peak appeared at 1500 hr, beside two peaks at 0600 hr and 2400 hr whereas the minimum activity emerged at 0900 hr (Fig. 4). The difference between maximum peak and minimum peak was significant ($P<0.01$).

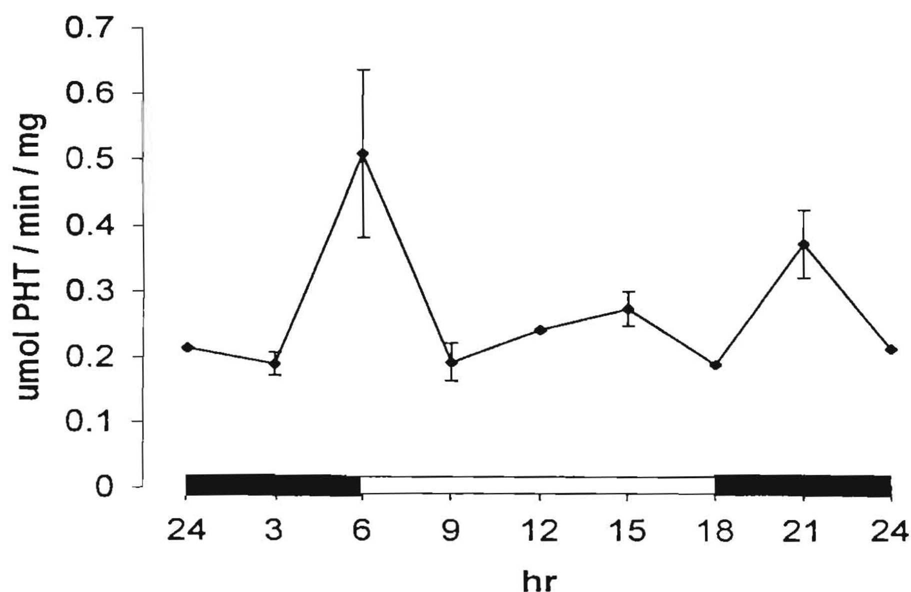

Fig 1 Circadian variation in hepatic aldehyde oxidose activity. Enzyme activity was measured at 37°C and expressed as m mol Phthalazine (PHT) oxidized/min/mg protein. Potassium ferricyanide (1 μM) was used as an electron acceptor. Each point represents the mean ±SE of six animals

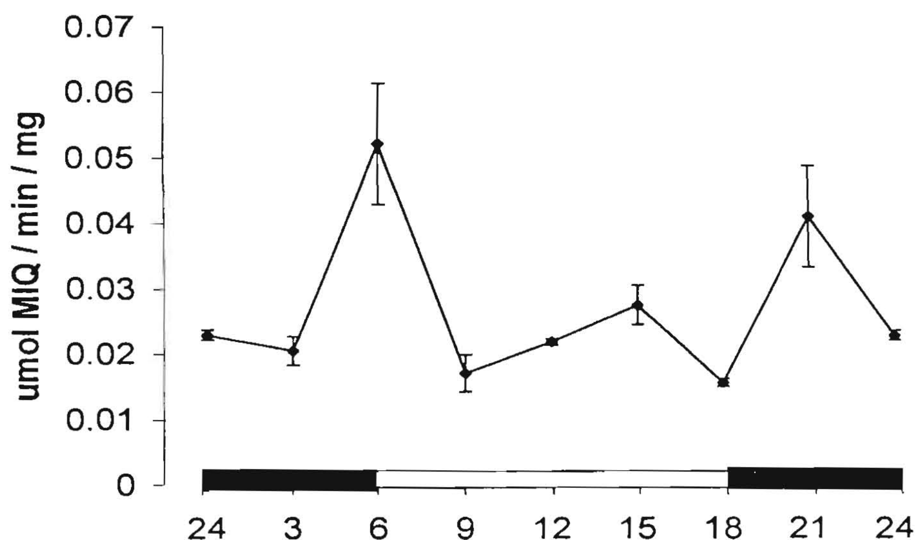

Fig 2 Circadian variation in hepatic aldehyde oxidase activity. Enzyme activity was measured at 37 ^{o}C and expressed as mmol 3-Methyliosoquinoline (MIQ) oxidized/min/mg protein. Potassium ferricyanide (1 μM) was used as an electron acceptor. Each point represents the mean ±SE of six animals

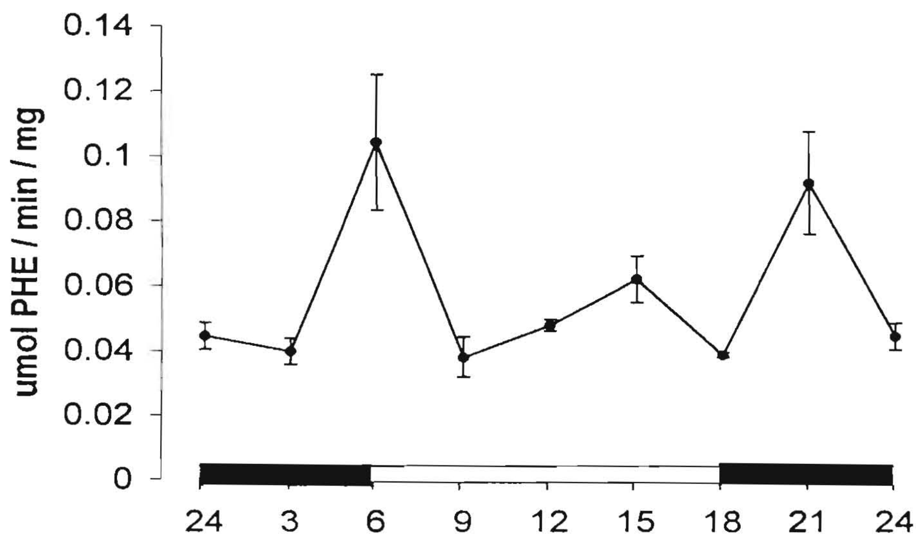

Fig 3 Circadian variation in hepatic aldehyde oxidase activity. Enzyme activity was measured at 37 ^{o}C and expressed as μmol Phenonthsidine (PHE) consumed/min/mg protein. Oxygen was used as an electron acceptor. Each point represents the mean ±SE of six animals

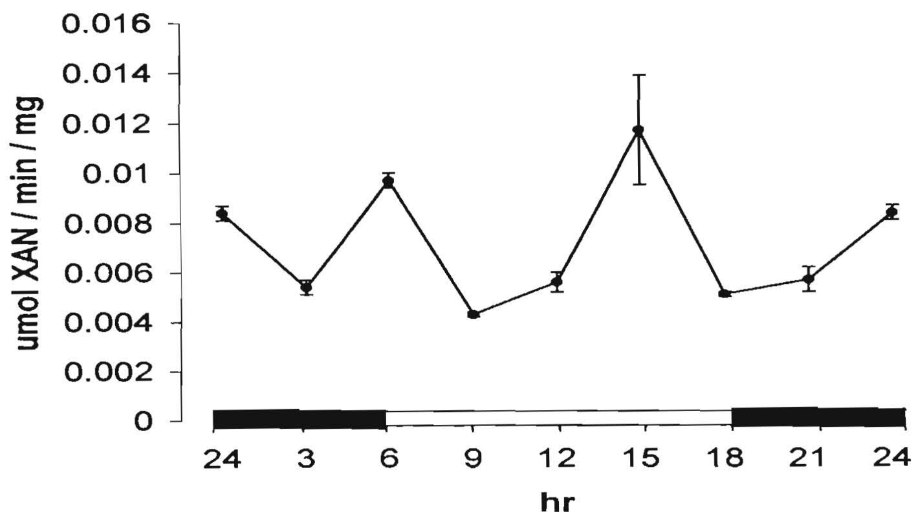

Fig 4 Circadian variation in hepatic aldehyde oxidase activity. Enzyme activity was measured at 37 ^{o}C and expressed as µmol Xanthine (XAN) oxidized/min/mg protein. Oxygen was used as an electron acceptor. Each point represents the mean ±SE of six animals

These results show that the activity of hamster molydbenum hydroxylases, aldehyde oxidase and xanthine oxidase, has daily variations, but comparing with guinea pig enzymes (Beedham *et al.* 1989), the fluctuation in the activity of hamster xanthine oxidase is more pronounced than the flucuation of xanthine oxidase in guinea pigs. Circadian rhythms have been observed for several drug metablising enzymes (Radzialowski and Bousquet, 1968; Lake *et al.* 1976; Wolfe and Schell, 1979; Birt and Hines, 1982; and Beedham *et al.* 1989). These rhythms have been attributed to many factors, including changes in corticosteroids, light dark-schedule and dietary protein (Radzialowski and Bousquet, 1968; Nair and Casper, 1969; and Birt and Hines, 1982).In addition, other workers have found that the daily rhythm in hepatic hexobarbital oxidase and O-demethylase hinges on environmental lighting. Exposure of rodents to either continuous illumination or darkness suppressed these rhythms (Holcslow *et al.* 1975; and Nair and Casper, 1969).Furthermore, these circadian cycles may be due, in part, to feeding times (Birt and Hines, 1982, and Beedham *et al.* 1989). Several factors regulate molybdenum hydroxylases activities *in vivo*. These include diet, induction and hormonal influences.

Rowe and Wyngaarden (1966) found that hepatic xanthine oxidase levels in rats varied with the amount of protein in the diet, with increased activity coupled with high protein diets and decreased levels during periods of protein insufficiency.

Specific induction of the molybdenum hydroxylases by xenobiotics has been established in only a few investigations. Oral administration of phthalazine or 1-hydroxyphthalazine to rabbits caused an increase in the activity of aldehyde oxidase and xanthine oxidase in liver (Johnson *et al.* 1984). Similarly, xanthine oxidase levels are elevated when mice are treated with xanthine (Dietrich, 1954). Beedham *et al.* (1989) found that exogenous administration of melatonin to guinea pigs caused a significant increase in hepatic aldehyde oxidase and xanthine oxidase. Furthermore, they have concluded that melatonin concentrations may be related to the circadian variation in liver molybdenum hydroxylase activity. Further support for the hormonal influences on molybdenum hydrxylases is provided in reports on some species including rats, mice and guinea pigs (Huff and Chaykin, 1976; Levinson and Decker, 1984; and Beedham *et al.* 1987b). These studies have shown a higher hepatic molybdenum hydroxyase activity in the male, and this increase in male enzymes has been ascribed to greater testosterone levels. Although males were not used in the present study, the results reinforce the findings of other researchers who have suggested that the molybdenum hydroxylases are subject to daily variation in guinea pigs (Beedham *et al.* 1989). In addition, these changes in the activity of molybdenum enzymes, particularly aldehyde oxidase, were synchronous with fluctuations in serum melatonin levels. Further study will establish whether aldehyde oxidase and xanthine oxidase from hamsters are dependent on the variable secretions of pineal melatonin.

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التغييرات اليومية الطارئة على نشاط المولبيدينوم هيدروكسيلزز الكبدي في إناث الهامستر السوري

يوسف عبدالملك الطيب

المستخلص: تم قياس نشاط إنزيمات المولبيدنوم هيدروكسيلزز (إنزيم الدهايد أوكسيديز وإنزيم الزانثينن أوكسيديز) والتي حُضرت بنقاوة، إلى حد ما، من أكباد إناث الهامستر البالغة ثماني مرات يومياً، عند فترات زمنية منفصلة ومتساوية. ثم تمت ملاحظة التغييرات اليومية المميزة التي تطرأ على نشاط الألدهايد أوكسيديز مع مواد يعمل عليها الإنزيم (الثالازين، مثيل ايزوكوينولين، الفينانثيرين).

وقد كانت قمة نشاط هذا الأنزيم عند الساعة السادسةصباحاً، وكان أدنى نشاط له عند الساعة الثالثة والتاسعة صباحاً والساعة الثامنة عشر مساء. وقد لوحظ وجود فروق معنوية بين قمة نشاط هذا الإنزيم ،القيم الدنيا له ($P<0.005$). ثم تمت ملاحظة التغييرات اليومية التي تطرأ على نشاط (الزانثين أوكسيديز) باستخدام (الزانثين) كمادة تفاعل. وكانت قمة نشاط هذا الانزيم عند الساعة الخامسة عشر ظهراً وأدناه عند الساعة التاسعة صباحاً، وكان الفرق بين أعلى وأدنى نشاط فرقاً معنوياً ($P<0.01$).

أوضحت نتائج هذه الدراسة أن إنزيمات الدهايد أوكسيديز والزانثين أوكسيديز الكبدية للهامستر ، قد طرأ عليها تغيير يومي على نشاطها.