JKAU: Sci., **vol. 16,** pp. 63-71 (2004 A.D. / 1424 A.H.)

Circadian Rhythms of Hepatic Molybdenum Hydroxylases Activities in the Rat

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ABSTRACT. The objectives of this study is to investigate the occurrence of circadian rhythms with molybdenum hydroxylases as many other enzymes were found to show this phenomenon. The specific activities of molybdenum hydroxylases, namely; aldehyde oxidase and xanthine oxidase were measured in partially purified ammonium sulfate fractions of adult rat liver eight times daily at equal intervals. The activity of aldehyde oxidase was found to vary markedly with the substrates used (3-methylisoquinoline and phthalazine). The highest enzyme activity with both substrates was observed at 1500 hr, whereas the lowest activity occurred at 0900 hr. There were highly statistically significant ($P < 0.0005$) differences between the maximum and minimum activities. Circadian variation was also observed in xanthine oxidase activity when xanthine was used as a substrate. Two main maximum peaks appeared at 1500 hr and 2100 hr, and the minimum activity peak was shown at 0300 hr. The difference between the rhythmic extremes was statistically significant ($P < 0.0005$). These results indicate that some factors such as hormones, may be responsible for the circadian variations exhibited in aldehyde oxidase and xanthine oxidase activities.

KEY WORDS: Aldehyde Oxidase; Xanthine Oxidase; Molybdenum Hydroxylases; Rat; Circadian Rhythms.

Introduction

Mixed function oxidases or microsomal monooxygenases are known to play a vital role in the biotransformation of a wide range of drugs and xenobiotics[1-5]. Moreover, the cystosolic molybdenum hydroxylases, namely, aldehyde oxidase (E.C. 1.2.3.1) and xanthine oxidase (E.C. 1.2.3.2) are also involved in the

metabolism of drugs and foreign compounds[6-9]. These enzymes are widely distributed throughout the animal kingdom from primitive species and majority of mammals[6]. In many animal species such as rats, mice, and guinea pigs the activity of these cytosolic enzymes appeared to show temporal variations. The existence of daily rhythmic variations in the activities of these enzymes is well documented. Variations in hepatic male rats were previously reported[10-12]. Radziolowski and Bouquet[10] used different substrates such as aminopyrine, p-nitroanisole, hexobarbital and 4-methylaminobenzene, and found that the circadian rhythm maximum activity occurred at 0200 hr, whereas the minimum activity was at 1400 hr, and similar result was obtained with females. The diurnal rhythm of hepatic O-dethylase and hexobarbital oxidase was also observed by Nair and Casper[11] after exposing rats to alternating periods of light and darkness. The maximum activity of O-dethylase was at 0200 hr and the lowest at 1800 hr whereas hexobarbital oxidase showed a maximal value at 2200 hr, and a minimal value at 1400 hr. Jori *et al.*[12], determined the activity of rat liver microsomal enzymes at different times of the day with different illumination periods using hexobarbital, imparine, p-nitroanisole and aminopyrine as substrates. They reported that in normal illumination conditions (6.30 a.m. to 6.30 p.m.) a pattern of a minimum microsomal enzyme activity was shown between 10.00 a.m. and 2.00 p.m. and another maximum activity during darkness. Furthermore, the activity of two transferase enzymes in rat liver was found to have diurnal variations[13]. Moreover, molybdenum hydroxylases were also found to display diurnal variation in guinea pig[14], and hamster[15] liver.

Due to lack of information regarding aldehyde oxidase and xanthine oxidase in rat liver, this work was conducted to investigate whether both enzymes could display circadian variations in their activity.

Materials and Methods

Chemicals

Phthalazine was supplied by Aldrich Chemical Company (Gillingham, U.K.). 3-Methylisoquinoline was purchased from ICN Pharmaceuticals Inc. (K&K) (Irvine, CA). Xanthine was bought from Sigma Chemical Company (Poole, U.K.).

Animals

Adult male Wistar rats weighing approximately 240 g each were used in this study (48 rats for each used substrate). They were obtained from King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. Rats were maintained on a standard laboratory diet (Oxoid, modified 41B) and

allowed to feed *ad libitum* with free access to water. They were housed in groups of 6, and kept in a regime of a strictly controlled temperature of (17 ± 1ºC) and a lighting cycle of 0600-1800 hr light and 1800-0600 hr dark for 2 weeks before experimentation. All animals were scarified by cervical dislocation at intervals of three hours for each group, and the livers were rapidly collected free of the gall bladder, frozen in liquid nitrogen, and kept in a deep-freezer at –80ºC for 3 weeks.

Hepatic Enzyme Preparation

Partially purified aldehyde oxidase was prepared according to the method described by Johnson *et al.*[16] with some modifications. The frozen livers were weighed, finely chopped and homogenized for 30 sec with a polytron homogenizer in 3 volumes of ice-cold potassium chloride solution (1.15 w/v) containing 0.1 mM EDTA. The resulting homogenate was heated on a water bath at 50-55ºC for 15 minutes, followed by immediate cooling to 10ºC in ice and then the suspension was centrifuged at 20,000 g for 25 minutes at 4ºC using a Heraeus Christ 20-3 (LABSCO) centifuge. The resulting supernatant was filtered through glass wool, and sufficient solid ammonium sulfate was then added to the filtered supernatant to give 50% saturation. The mixture was stirred for 15 minutes on an ice bath. After the precipitation was completed, the suspension was centrifuged at 3000 g for 15 minutes at 4ºC. The precipitate was rinsed with distilled water and dissolved in 5 ml of 0.1mM EDTA. The hepatic partially purified enzyme was stored in deep freezer at –80ºC until required for spectrophotometric assays.

Enzyme Assays

Due to the multiple forms (isozymes) of molybdenum hydroxylases, different substrates were used to monitor the enzyme activity. The specific activity of partially purified aldehyde oxidase was determined using a Varian UV/VIS spectrophotometer at 37ºC in 67 mM phosphate buffer pH 7 as previously reported by Johnson *et al.*[17]. The oxidation rate of either phthalazine (1 mM) or 3-methylisoquinoline (1 mM) was evaluated at 420 nm indirectly by observing the reduction of potassium ferricyanide as an artificial electron acceptor, while the specific activity of xanthine oxidase was estimated with xanthine (50 uM) at 295 nm as described by Johnson *et al.*[17]. The concentration of protein was determined using Biuret method.

Statistical Analysis

The data presented in this report are expressed as mean ± SE of six animals for each point of the enzyme activity of each substrate. Student *t* test was used

to determine the significance of the difference between the maximum and minimum values.

Results and Discussion

Several studies showed that the interaction of aldehyde oxidase with N-heterocyclic compounds varies markedly from one species to another[18-21], but the daily activity of molybdenum hydroxylases was only investigated in guinea pig and recently in hamster[14-15]. The present work aimed to determine the activity of rat aldehyde oxidase and xanthine oxidase at 3 hourly intervals during a period of 24 hr under controlled lighting cycle. Figures 1 and 2 show the circadian variations of hepatic aldehyde oxidase activity using phthalazine and 3-methylisoquinoline indirectly at 420 nm by observing the reduction of potassium ferricyanide as an artificial electron acceptor. The activity of aldehyde oxidase with phthalazine appeared to have two peaks as displayed in Figure 1. A maximum peak was in the light phase (1500 hr) and an intermediate peak at 2100 hr whereas the minimum activity was observed at 0900 hr and 1800 hr. Figure 2, also shows a maximum peak at 1500 hr and another intermediate on at 2100 hr in the dark phase. The minimum aldehyde oxidase activity with 3-methylisoquinoline was found to be 0900 hr. Both figures show more or less similar pattern of maximum and minimum activities. The difference between maximum and minimum peaks with both substrates were highly significant ($P < 0.0005$). The occurrence of isozymes of aldehyde oxidase in rat and mouse was previously reported by Holmes[22]] and Ohkubo *et al.*[23], and the present results suggest that the two substrates are catalyzed by the same form of aldehyde oxidase[15].

The activity of xanthine oxidase exhibited a markedly different circadian variation. Figure 3 shows two maximum peaks at 1500 hr and 2100 hr, whereas the minimum peak was 0900 hr. The difference between the maximum and minimum peaks was also significant ($P < 0.0005$).

The data in the present study proves that the activity of hepatic rat aldehyde oxidase and xanthine oxidase has circadian variations. Two studies carried out by Beedham *et al.*[14] and Al-Tayib[15] found that the activity of aldehyde oxidase and xanthine oxidase showed daily variations in guinea pig and hamster although the activity of xanthine oxidase in hamster was more fluctuated than in guinea pig.

In conclusion, circadian variations which have been found to occur in the activity of molybdenum hydroxylases of several rodents could be attributed to different factors including hormonal levels[12,13,24], and probably other factors need to be investigated. These results on rats indicate that circadian rhythms in the

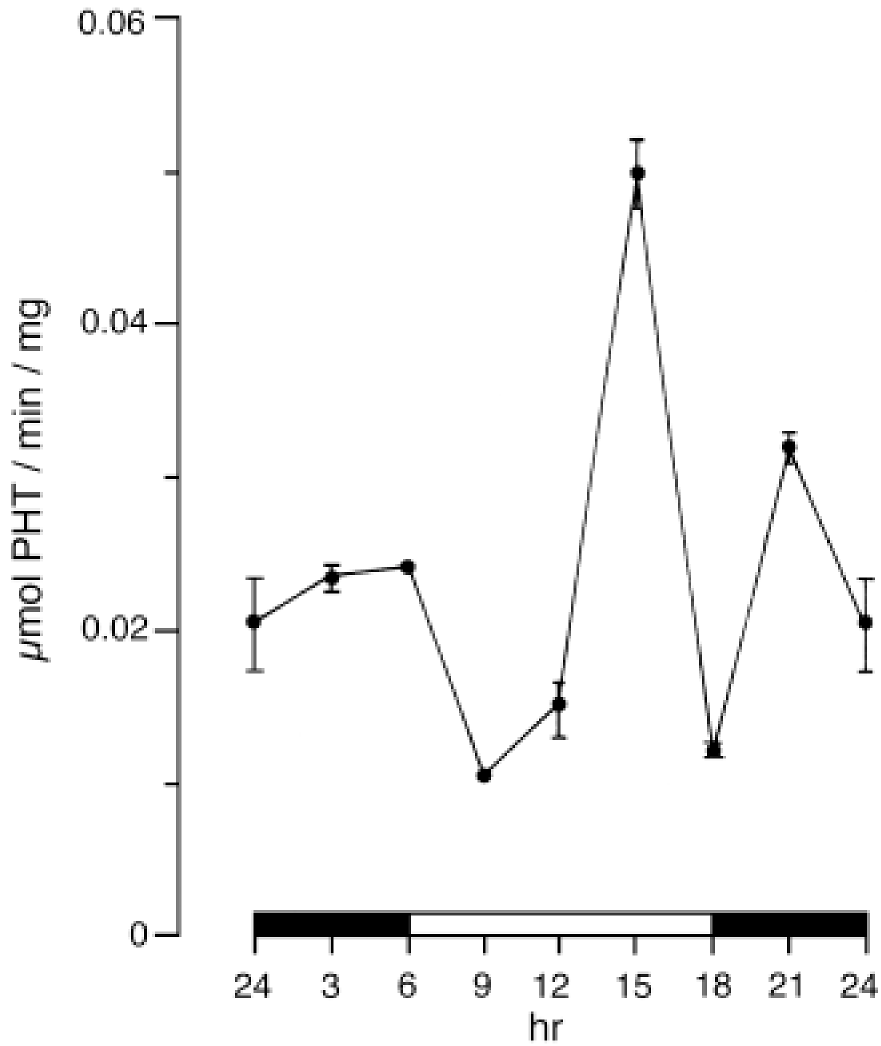

FIG. 1. Circadian variation of hepatic aldehyde oxidase activity. Enzyme activity was measured at 37ºC and expressed as µmol phthalazine (PHT) oxidized/min/mg of protein. Each point represents the mean ± of six animals.

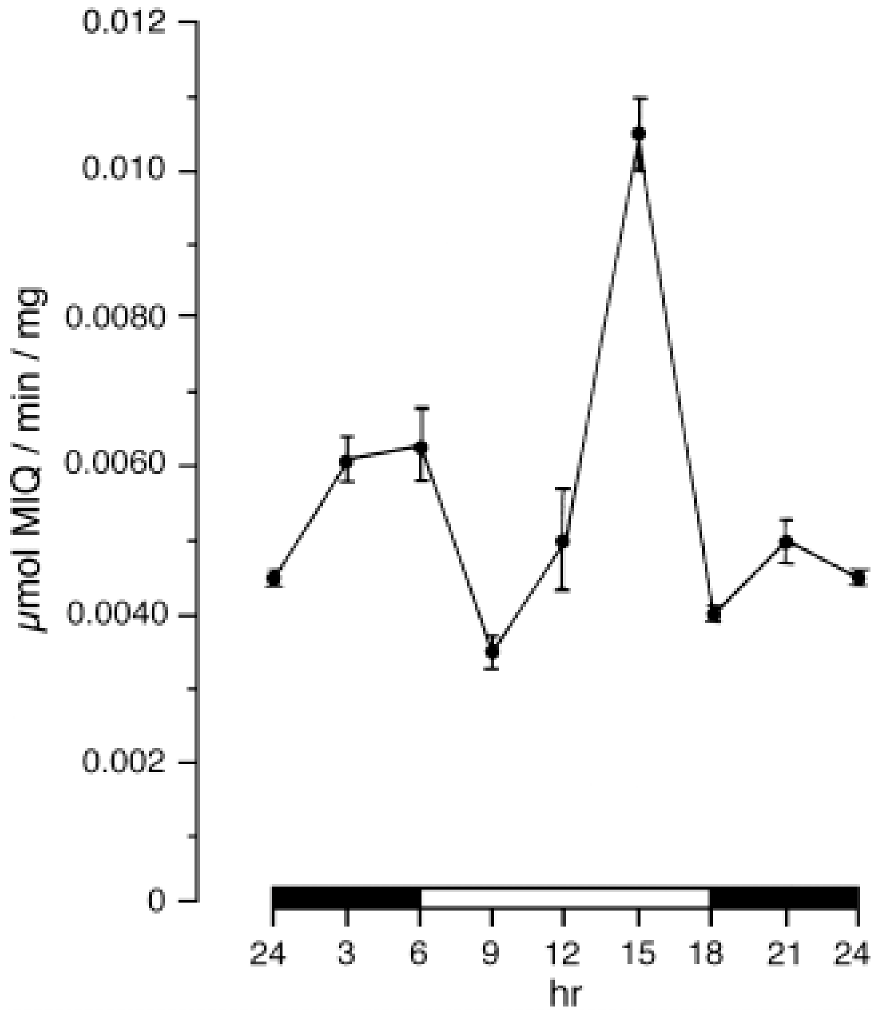

FIG. 2. Circadian variation of hepatic aldehyde oxidase activity. Enzyme activity was measured at 37ºC and expressed as µmol 3-methylisoquinoline (MIQ) oxidized/min/mg protein. Each point represents the mean ± SE of six animals.

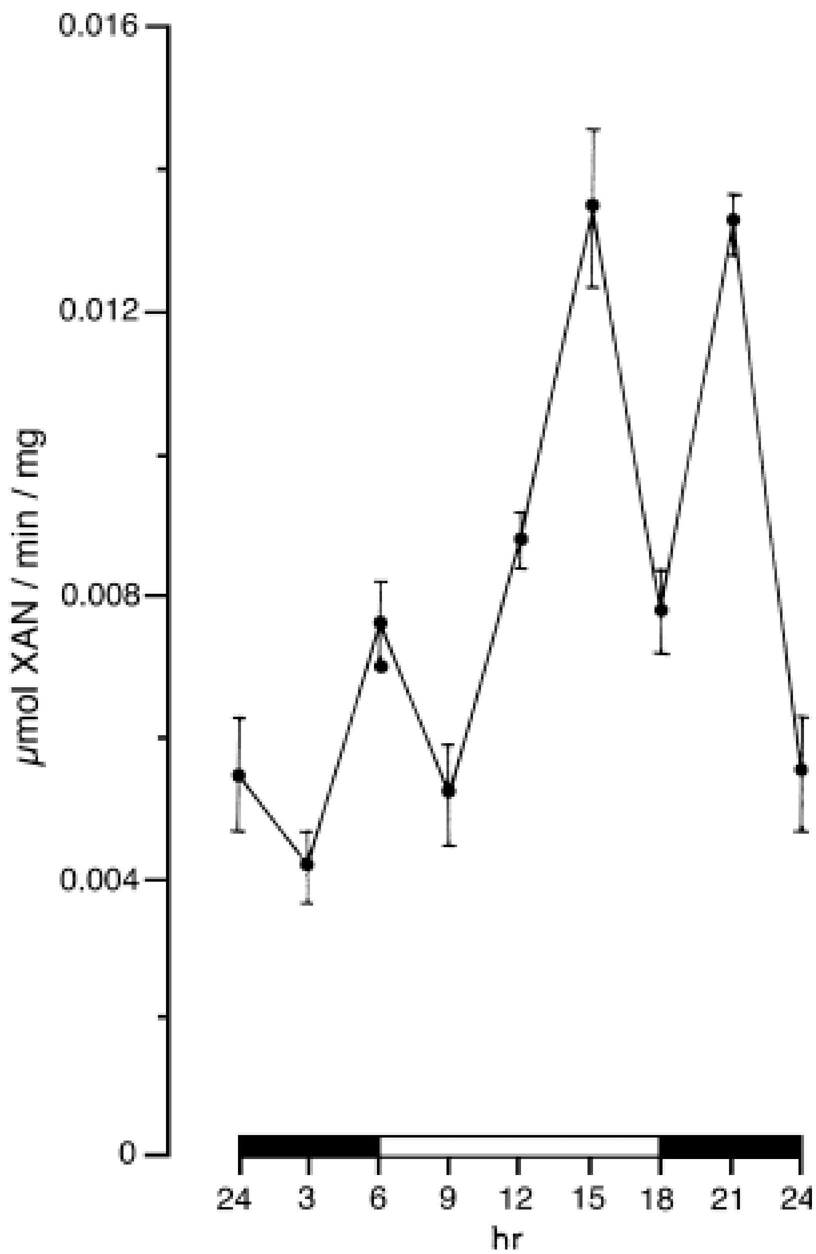

FIG. 3. Circadian variation of hepatic xanthine oxidase activity. Enzyme activity was measured at 37ºC and expressed as μmol xanthine (XAN) oxidized/min/mg protein. Each point represents the mean ± SE of six animals.

metabolism of various drugs by the two molybdenum hydroxylases are well correlated with previous studies using the mammals as well as unicellular algae[25].

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الإيقاعات اليومية الحادثة على نشاطية إنزيمات موليبدينوم هيدروكسيليزز فى الجرذ

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المستخلص. هدف الدراسة هو التحقق من حدوث إيقاعات يومية لإنزيمات موليبدينوم هيدروكسيليزز نظراً لأن إنزيمات أخرى كثيرة وجد أن لديها هذه الظاهرة . تم تقدير نشاط إنزيمات موليبدينوم هيدروكسيليزز (ألدهايد أوكسيديز وزانثين أوكسيديز) المحضرة بنقاوة جزئية من أكباد ذكور الجرذان البالغة ثماني مرات يوميًا على فترات زمنية متساوية وذلك باستخدام اثنين من مواد تفاعل ألدهايد أوكسيديز (٣-ميثايل أيزوكينولين وثالازين) لقياس نشاط الإنزيم. وقد أوضحت النتائج مع كلتا المادتين حدوث قمة نشاط الإنزيم عند الساعة الثالثة ظهراً وكان أدنى نشاط عند الساعة التاسعة مساءً بينما كان الفرق بين أعلى و أدنى نشاط معنوي هو ($P < 0.0005$) كذلك أوضحت النتائج على نشاط زانثينً أوكسيديز باستخدام زانثين كمادة تفاعل أعلى نشاط لهذا الإنزيم عند الساعة الثالثة ظهراً و التاسعة مساءً بينما كان الفرق بين أعلى و أدنى نشاط معنوي هو ($P < 0.0005$) تدل نتائج الدراسة على أن بعض العوامل مثل الَهرمونات قد تكون مسئوله عن التغيرات اليومية على نشاط ألدهايد أوكسيديز و زانثين أوكسيديز فى كبد الجرذ .