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Long-term variation in basal and phenobarbital-stimulated oxidative drug metabolism in the rat

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SEVERAL studies have shown that responses to drugs may vary as a function of time in a rhythmic manner. We have demonstrated an endocrine-dependent circadian rhythm in oxidative drug metabolism in the rat and mouse;[1] this has recently been confirmed by Vesell[2] and by Colas *et al.*[3] Similar rhythms in drug responsiveness or toxicity or in both have been shown for morphine,[4] pentobarbital,[5] nikethamide[6] and lidocaine.[7] Longer term fluctuations, known as seasonal or circannual rhythms and having a 12-month base, are present in a variety of physiologic and biochemical functions. Ankier *et al.*[8, 9] report the presence of a seasonal dependence in the rat to the anaphylactic properties of dextrans and other antigens with a winter peak in mortality. Nayler[10] has demonstrated a seasonal rhythm in cardiac phosphorylase activity in the toad with summer values of about 60 per cent of the winter value, while Kennedy and Nayler[11] have shown that summer levels of magnesium-dependent, sodium- and potassium-activated ATPase in toad cardiac muscle are about 30 per cent of winter values. A striking example of seasonal dependence in a response of pharmacologic interest is afforded by the studies of Fearn *et al.*,[12] who examined the blood pressure response of the rat to histamine at different times of the year. During the period September–January, 10–50 μg/kg doses of histamine evoked an average depressor response of 40 mm Hg; from February to April, histamine at 100–500 μg/kg elicited an average depressor response of 20 mm Hg, whereas during the period May–August doses of histamine from 1 to 5 mg/kg failed to modify blood pressure. In a study employing 28,000 mice over a 3½-yr period, Sterne and Hirsch[13] have demonstrated a marked reduction in the lethality of dimethylguanylguanidine, a non metabolized hypoglycemic agent, during the summer months, which appears to relate to a seasonal susceptibility to the effects of hypoglycemia. Kalser and Kunig[14] have recently suggested the presence of a seasonal dependence in the response of Wistar rats to hexobarbital hypnosis. In our own laboratory,* an apparent seasonal variation in the lethality of drugs such as morphine and insulin in the rat has been noted.

Observations of this type suggest that in studies of long duration drug responses may be measured against a constantly changing baseline and that significant qualitative and quantitative alterations may be expected. The specific objectives of the preliminary experiments to be reported were: (1) to determine if seasonal rhythmicity in oxidative drug metabolism is evident in the rat; (2) to determine if rhythmicity is present in the response of this species to enzyme-stimulating drugs such as phenobarbital; and (3) to attempt a correlation of long-term alterations in drug metabolism with drug response measured under *in vivo* conditions.

Male, Holtzman rats, weighing between 135 and 150 g at the time of use, were employed as experimental animals. Animals were maintained on wire-mesh flooring in the laboratory for at least 5 days after receipt from the supplier prior to use and had free access to water and commercial chow.

*D. E. Blake, D. R. Haubrich and J. E. Thornburg, unpublished observations.

Experiments were carried out between the 14th and 17th days of each month between 8 and 10 a.m. Hexobarbital sleeping times were measured at an ambient temperature between 76 and 78°F. Groups of six to ten animals were challenged with hexobarbital sodium (100 mg/kg), i.p.) 48 hr after receiving either saline (1 ml/kg) or phenobarbital sodium (100 mg/kg). The duration of loss of the righting reflex was measured. Other groups of six animals, which had received either saline or phenobarbital sodium as above, were sacrificed by cervical dislocation and the livers were rapidly removed, blotted and placed upon crushed ice. Livers were minced individually (Harvard tissue press) and homogenized in 3 vol. of 0·1 M phosphate buffer, pH 7·4, for 1 min. Homogenates were centrifuged at 10,000 rpm in a Spinco model L2-50 ultracentrifuge (9000 g_{av}, no 30 rotor) for 20 min. The supernatant fractions were employed in subsequent enzyme assays. The metabolism of each substrate was measured in the same enzyme pool from each animal. Protein was determined by the procedure of Lowry *et al.*[15] with bovine serum albumin as a standard.

Incubation flasks contained 1·0 ml (250 mg liver equivalent) of the 9000 *g* supernatant fractions, 1·0 ml of 0·1 M phosphate buffer, 30 μmoles glucose 6-phosphate, 50 μmoles nicotinamide, 50 μmoles

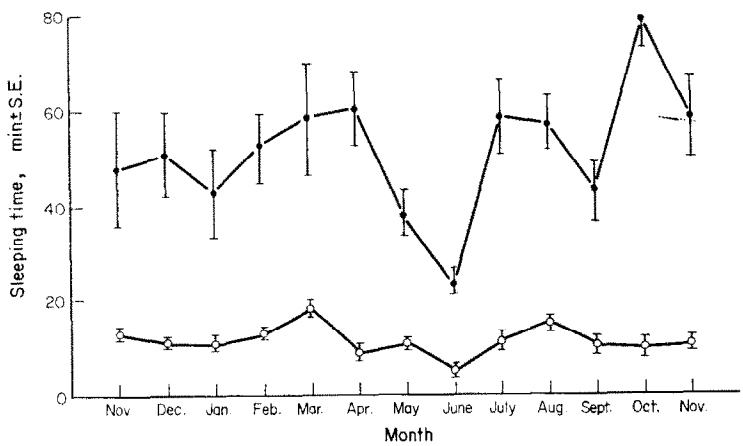

FIG. 1. Hexobarbital sleeping times in control (● —— ●) and phenobarbital-pretreated (○ —— ○) rats. Six to ten animals per group.

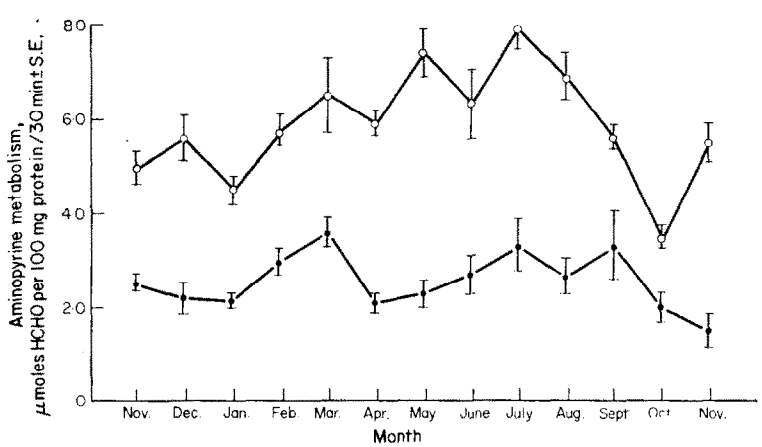

FIG. 2. Aminopyrine demethylation by 9000 *g* fractions of liver from control (● —— ●) and phenobarbital-pretreated (○ —— ○) rats; six animals per group.

magnesium chloride and 4 μmoles of NADP in a final volume of 5·0 ml. Semicarbazide (45 μmoles) was added to flasks in which aminopyrine was the substrate. Levels of substrates were, respectively: aminopyrine, 10 μmoles; hexobarbital, 4 μmoles; and *p*-nitroanisole, 10 μmoles. Reaction mixtures containing hexobarbital or *p*-nitroanisole were incubated at 37° for 20 min and a 30-min incubation was used for aminopyrine-containing flasks. In each instance reactions were shown in preliminary studies to be linear during these time periods. Formaldehyde formed from aminopyrine demethylation was determined by the method of Cochin and Axelrod,[16] unmetabolized hexobarbital by the technique of Cooper and Brodie,[17] and *p*-nitrophenol formation by the procedure of Netter and Seidel.[18]

Data presented in Fig. 1 show the variation in responsiveness of the rat to hexobarbital and the modification of hexobarbital sleeping time by phenobarbital pretreatment over a 1-yr period. There exists a variation of approximately 4-fold in the sleeping times of controls during this period, whereas the response to hexobarbital in the phenobarbital-pretreated animal is much more uniform. The apparent response to the enzyme-inducing action of phenobarbital, viewed as a function of the

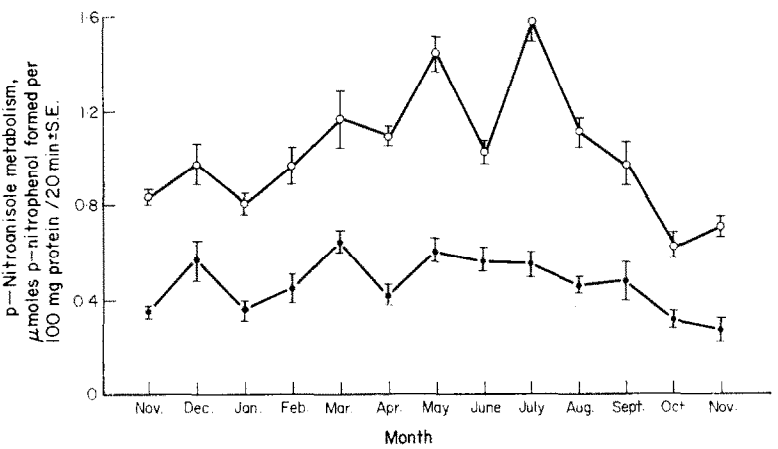

FIG. 3. *O*-dealkylation of *p*-nitroanisole by 9000 *g* fractions of liver from control (● —— ●) and phenobarbital-pretreated (○ ——○) rats; six animals per group.

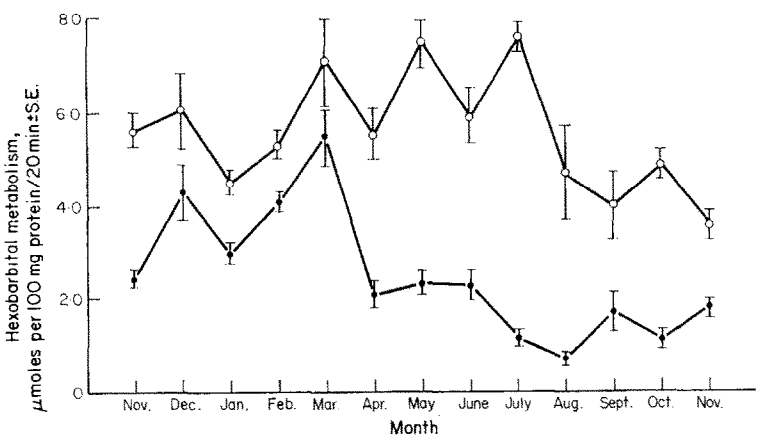

FIG. 4. Hexobarbital oxidation by 9000 *g* fractions of liver from control (● —— ●) and phenobarbital-pretreated (○ —— ○) rats; six animals per group.

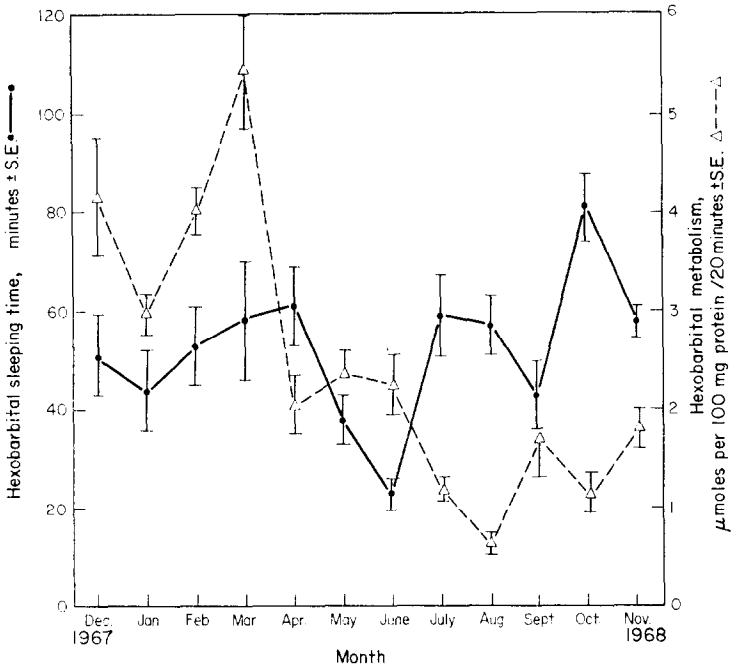

FIG. 5. Hexobarbital oxidation by 9000 *g* fractions of rat liver (△ - - - - △) and sleeping time after administration of 100 mg/kg, i.p., of hexobarbital sodium (● —— ●).

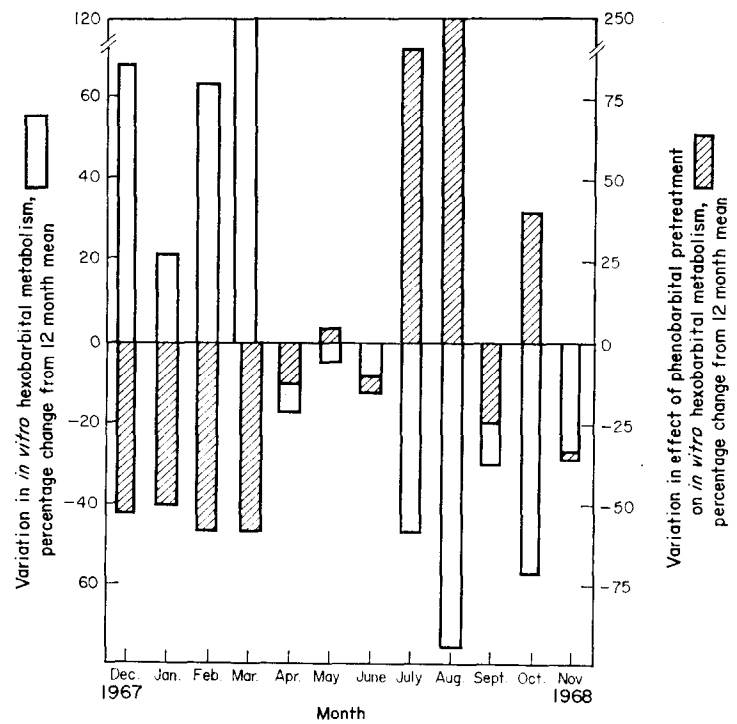

FIG. 6. Monthly deviation in relation to the 12-month mean of hexobarbital oxidation by 9000 *g* fractions of liver from control (▭) and from phenobarbital-pretreated (▨) animals.

appropriate "control" group, exhibits substantial variation. The ratio of sleeping time responses between these two groups is about 4·5 in June and 8·0 in October. This is suggestive of a greater sensitivity to the enzyme-stimulating properties of phenobarbital during the summer months. This is further demonstrated when the metabolism *in vitro* of the three substrates is examined in phenobarbital-pretreated animals as shown in Figs. 2–4. In each case a summer peak in responsiveness to the barbiturate is evident. Figure 5 represents the relationship between hexobarbital sleeping time and metabolism *in vitro* of the barbiturate over the 12-month period. Of particular interest is the poor apparent correlation between the direction of change of these two parameters. During the first 5 months of the study, each point on the metabolism curve is significantly different ($P < 0{\cdot}05$) from that preceding and following it, while none of the sleeping time responses is significantly different ($P < 0{\cdot}3$) from another. These data reemphasize the need for critical and independent verification of results from studies *in vitro* and *in vivo*, with minimal inference being drawn regarding causal interrelationships.

When the variation in hexobarbital metabolism measured under conditions *in vitro* in control and phenobarbital-pretreated rats is plotted with respect to the 12-month mean, two factors become apparent, as shown in Fig. 6. First, the presence of a rhythm in basal levels of hexobarbital oxidase is suggested, and second, the phenobarbital effect is clearly related to the basal enzyme activity at the time of phenobarbital administration. Thus, when basal enzyme levels are high (December–March) the stimulation of hexobarbital oxidation by phenobarbital pretreatment is lowest. Conversely, when basal levels of hexobarbital oxidase are minimal (July–August, October), response to the phenobarbital is greatest. The apparent summer peak in response to enzyme induction by phenobarbital is shown for the three substrates in Fig. 7.

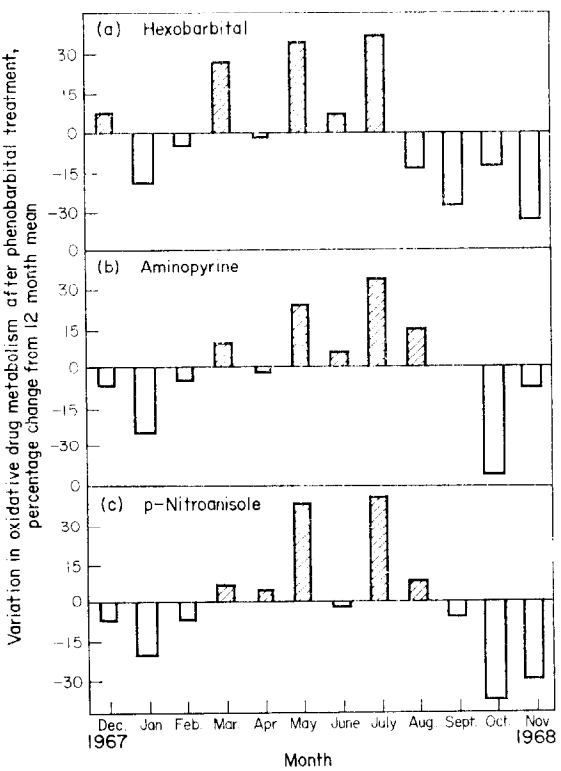

FIG. 7. Monthly deviation in relation to the 12-month mean of hexobarbital, aminopyrine and *p*-nitroanisole metabolism by 9000 *g* fractions of rat liver after phenobarbital treatment.

Although continuing studies over a 2- or 3-yr period will be necessary to substantiate certain of the preliminary observations reported here, the present data suggest that a seasonal or circannual rhythmicity exists in oxidative drug metabolism and in the stimulation of oxidative drug metabolism by phenobarbital pretreatment in the rat. The presence of a summer peak in responsiveness of the rat to the enzyme-stimulating effect of phenobarbital was seen both in the metabolism *in vitro* of the model substrates (hexobarbital aminopyrine and *p*-nitroanisole) and in the shortening of hexobarbital sleeping time. These findings suggest that the time of year may be an important variable in the evaluation of the enzyme-stimulating properties of drugs and other foreign compounds. Chronic toxicity studies with compounds able to enhance their own metabolism upon repeated administration could conceivably be influenced by seasonal differences in sensitivity to enzyme stimulation.

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