Pregnancy Increases Reactivity of Mice to Phenobarbital

Abstract. *Compared to nonpregnant controls, pregnant mice injected with phenobarbital had lower concentrations of the drug in the plasma but equivalent concentrations in the brain. In spite of the similar concentrations in the brain, the behavioral response to phenobarbital was greater for pregnant than nonpregnant mice. These results suggest that the concentration of phenobarbital in the plasma, which is commonly used as a basis for adjusting phenobarbital dosage during pregnancy, is not an appropriate indicator of the dynamics of the drug.*

Phenobarbital is used by pregnant women as a sedative (*1, 2*), a prophylaxis for neonatal hyperbilirubinemia (*3*), and an anticonvulsant (*2, 4, 5*), even though there are indications that the drug may be teratogenic (*5*). Studies in laboratory animals, for example, indicate that maternal exposure to phenobarbital produces offspring that are suboptimal on tests of behavior (*6–8*), endocrine function (*9, 10*), and neurochemistry (*7, 8, 11*). These studies, at the very least, indicate that the drug should be used cautiously during pregnancy and administered at a minimum therapeutic dose. However, plasma concentrations of phenobarbital decline during this period and the dose is frequently increased for epileptics (*12*), even though pregnant epileptics sometimes remain asymptomatic (*13*) and the need for maintaining prepregnancy plasma concentrations of the drug is not empirically well established. Indeed, the present study on mice indicates that although pregnant animals have lower plasma concentrations of phenobarbital than nonpregnant animals, brain concentrations of the drug are relatively unaffected by pregnancy. The data also suggest that pregnant mice are more responsive to the drug than nonpregnant animals.

For this study, C57BL/6J mice were maintained on a cycle of 12 hours of light and 12 hours of darkness and were bred according to previously described procedures (*7*). Controls were mice not impregnated during the breeding procedure. Two experiments were conducted 12 days after a vaginal sperm plug was detected in the pregnant animals. Saline or sodium phenobarbital (20 mg, 40 mg, or 80 mg per kilogram of body weight) was injected subcutaneously (0.01 ml per gram of body weight). These doses produced plasma values (Table 1) well within the ranges reported for studies in humans. For example, in a recent report, 30 and 40 percent of the epileptics sampled had plasma phenobarbital concentrations of 8 to 15 μg/ml and 20 to 32 μg/ml, respectively (*14*).

In the first experiment, the locomotor activity of 32 pregnant ($N = 8$) and 32 nonpregnant ($N = 8$) mice was individually determined as previously described (*15*). The animals were tested for 60 minutes between 0800 and 1300 hours beginning within 60 seconds of the injection. The data from this experiment (see Fig. 1) were subjected to analyses of variance (*16*), with the locomotion of pregnant and nonpregnant mice being compared across time. The locomotion of pregnant and nonpregnant mice injected with saline (Fig. 1A) or with the 20 mg/kg dose of phenobarbital (Fig. 1B) was similar across time. The locomotion of pregnant mice differed from that of nonpregnant mice after the 40 mg/kg dose [Fig. 1C, pregnant versus time: $F(3, 42) = 4.558$, $P < .01$] and the 80 mg/kg dose of phenobarbital; [Fig. 1D, pregnancy: $F(1, 14) = 4.648$, $P < .05$].

The effect of pregnancy on response to phenobarbital was further characterized by comparing the activity scores of drugged mice with that of their saline controls (*17*). Compared to their respective saline controls, the drug-injected pregnant mice differed from their nonpregnant counterparts either by the absence of increased activity soon after injection (with the 40 mg/kg dose) or by earlier and more extensively reduced activity following injections of the 20, 40, and 80 mg/kg doses. Nonpregnant mice showed significantly elevated activity during the first or second 15-minute intervals after they received the 80 mg/kg (82 percent) or the 40 mg/kg dose (78 percent). In contrast, the activity of pregnant mice was not significantly increased at any of the drug doses or time periods tested. Nonpregnant mice had significantly reduced activity during the final period of measurement after the injection of the 80 mg/kg dose (−94 percent). In contrast, pregnant mice showed significantly reduced activity beginning with the second 15-minute interval for the 80 mg/kg dose (−58 percent); the third 15-minute interval for the 40 mg/kg dose (−53 percent); and the fourth 15-minute interval for the 20 mg/kg dose (−51 percent).

The greater reduction in the activity of pregnant compared with nonpregnant mice treated with phenobarbital indicates that pregnancy increased the response to this drug. This is in contrast to the reported effects of sodium pentabarbital, which depressed respiratory and cardiovascular systems less extensively in pregnant compared with nonpregnant female rats. The duration of drug action and time for drug clearance, however, was increased in pregnant animals (*18*). A further comparison of the drugs would be desirable with attention to species and time differences.

Because altered drug availability at critical sites could conceivably account for the observed behavioral difference, we conducted a second experiment to determine plasma and brain concentrations of the phenobarbital in pregnant and nonpregnant mice at 15, 30, 60, and 120 minutes after injection of either 20 or 80 mg/kg (*19*). These data (and the numbers of mice per group) are summarized in Table 1. Since the two doses produced large and obvious differences in plasma and brain levels, data within each dose were subjected to separate analyses of variance across the two pregnancy conditions and four time periods. The plas-

Table 1. Plasma and brain concentrations of phenobarbital in pregnant and nonpregnant mice at minute intervals after subcutaneous injections of 20 mg or 80 mg of sodium phenobarbital per kilogram of body weight. The data are expressed as means ± standard error of the mean.

Interval (min)	Plasma concentration (μg/ml)				Brain concentration (μg/g)			
	Nonpregnant mice	N*	Pregnant mice	N*	Nonpregnant mice	N*	Pregnant mice	N*
	Mice receiving the 20 mg/kg dose							
15	4.73 ± 0.20	7	4.17 ± 0.62	5	8.92 ± 0.67	7	8.08 ± 0.49	5
30	5.51 ± 0.14	7	4.60 ± 0.14	6	14.46 ± 0.59	7	14.76 ± 1.30	6
60	4.66 ± 0.15	6	3.93 ± 0.11	6	14.05 ± 0.54	6	13.87 ± 0.53	6
120	4.53 ± 0.14	6	4.21 ± 0.31	6	13.84 ± 0.75	6	13.87 ± 0.26	6
	Mice receiving the 80 mg/kg dose							
15	18.33 ± 0.41	7	14.46 ± 0.37	6	35.73 ± 1.54	7	34.86 ± 0.83	6
30	20.32 ± 0.51	7	14.09 ± 0.31	5	51.35 ± 0.98	8	45.93 ± 1.22	5
60	17.43 ± 0.38	5	14.44 ± 0.39	4	50.59 ± 2.30	6	52.25 ± 0.90	5
120	15.71 ± 0.85	6	13.74 ± 0.57	6	50.00 ± 1.87	6	50.36 ± 1.51	6

*Number of samples per group.

ma concentration of phenobarbital was lower for pregnant than nonpregnant mice for both the 20 mg/kg dose [$F(1, 42) = 7.578$, $P < .05$] and the 80 mg/kg dose [$F(1, 41) = 108.16$, $P < .01$]. In addition, plasma concentrations produced by the latter dose varied significantly across time for nonpregnant [$F(3, 41) = 6.29$, $P < .01$] but not for pregnant mice. Plasma drug concentrations of the drug in nonpregnant animals peaked at 30 minutes for both doses and was reduced by 20 percent from this value by 2 hours. In contrast, pregnant mice injected with 80 mg/kg had essentially constant plasma concentrations across the 2-hour sampling period, and these concentrations were consistently below those of nonpregnant mice. The lower plasma drug concentrations in pregnant mice is consistent with reports on pregnant women and may be accounted for by one or a combination of several factors, including changes in absorption, metabolism, or binding to plasma proteins which increase during pregnancy (*12*). In addition, since phenobarbital crosses the placenta, absorption by the placenta and fetus would reduce concentrations of the drug in the maternal plasma.

In contrast to the large reduction in plasma concentrations of the drug in pregnant mice, brain concentrations of phenobarbital were relatively unaffected by pregnancy. Pregnant and nonpregnant mice did not differ in peak brain concentrations for either dose; however, there was a trend toward a later peak for pregnant mice injected with the 80 mg/kg dose (*20*).

The lower concentrations of phenobarbital in the plasma of pregnant compared with nonpregnant mice did not reflect a similar difference in brain concentrations and is unlikely to account for the increased behavioral response to the drug. The increased response of pregnant mice to phenobarbital occurred in the absence of differences in whole brain concentrations. Thus, other factors, such as changes in regional drug distribution or compartmentalization, or changes in receptor sensitivity, must account for the increased response. These in turn may be associated with the animal's hormonal state which changes extensively during pregnancy. For example, estrogen, which increases substantially during pregnancy, facilitates the anticonvulsant activity of phenobarbital in nonpregnant female mice (*21*) and thus may also account for the increased response of pregnant mice to phenobarbital. Corticosterone also increases during pregnancy (*22*), and the interaction of

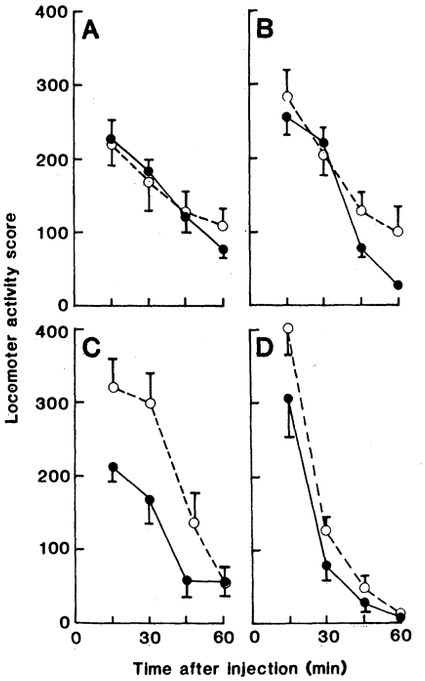

Fig. 1. Locomotor activity of (●) pregnant and (○) nonpregnant C57BL/6J mice at 15-minute intervals after subcutaneous injections of (A) saline or (B) 20 mg, (C) 40 mg, or (D) 80 mg of phenobarbital per kilogram of body weight. Points are averages from eight mice and bars are standard errors of means which extend beyond the symbol.

steroids with barbiturates is well established (*23*). It should be noted, however, that pregnancy-related differences in response to the shorter acting pentabarbital appear to be unrelated to hormonal changes and consistent with differences in drug concentrations in plasma and brain (*18*).

The present study demonstrates that, as noted for humans, plasma concentrations of phenobarbital per given dose are lower in pregnant than nonpregnant mice. It further demonstrates, however, that the reduced plasma concentrations during pregnancy are not accompanied by similar reductions in brain concentrations of the drug; and that, in spite of having brain concentrations similar to those in nonpregnant mice, pregnant mice are behaviorally more responsive to phenobarbital. Thus, an indicator other than plasma concentration may be appropriate for assessing whether or not phenobarbital dosage should be adjusted during pregnancy.

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References and Notes

1. J. O. Forfar and M. N. Nelson, *Clin. Pharmacol. Ther.* **14**, 632 (1973).
2. R. M. Hill, *ibid.*, p. 654.
3. C. Y. Yeung, L. S. Tam, A. Chan, K. H. Lee, *Pediatrics* **48**, 372 (1971); T. Vales, K. Koporous, S. Petmezaki, M. Solman, S. A. Doxiadas, *Pediatr. Res.* **14**, 947 (1980).
4. M. M. Desmond, R. P. Schwaneke, G. S. Wilson, S. Yasunage, I. Burgdorff, *J. Pediatr.* **80**, 190 (1972).
5. G. D. Montouris, G. M. Fenichel, L. W. McClain, Jr., *Arch. Neurol.* **36**, 601 (1979).
6. L. D. Middaugh, C. A. Santos, J. W. Zemp, *Dev. Psychobiol.* **8**, 305 (1975); *Pharmacol. Biochem. Behav.* **3**, 1137 (1975).
7. L. D. Middaugh, T. N. Thomas, L. W. Simpson, J. W. Zemp, *Neurobehav. Toxicol.* **3**, 271 (1981).
8. ———, *Psychopharmacology* **74**, 349 (1981).
9. C. Gupta, B. R. Sonawane, S. J. Yaffe, B. H. Shapiro, *Science* **208**, 508 (1980); C. Gupta, S. J. Yaffe, B. H. Shapiro, *ibid.* **216**, 640 (1982).
10. L. D. Middaugh, W. O. Boggan, C. Wilson-Burrows, J. W. Zemp, *Life Sci.* **24**, 999 (1981).
11. J. Yanai and J. J. Feigenbaum, *IRCS Med. Sci.* **9**, 965 (1981).
12. L. Bossi *et al.*, in *Antiepileptic Therapy: Advances in Drug Monitoring*, S. I. Johannessen *et al.*, Eds. (Raven, New York, 1980), p. 9; C. M. Lander, V. E. Edwards, M. J. Eadie, J. H. Turner, *Neurology* **27**, 128 (1977).
13. R. M. Hill, in *Epilepsy, Pregnancy and the Child*, D. Janz, Ed. (Raven, New York, 1982), p. 163.
14. C. M. MacLeod, A. S. Dekaban, E. Hunt, *Science* **202**, 1102 (1978).
15. L. D. Middaugh, L. A. Blackwell, W. O. Boggan, J. W. Zemp, *Pharmacol. Biochem. Behav.* **15**, 723 (1981).
16. Data were initially analyzed with a 2 (pregnancy) × 4 (dose) × 4 (time) analysis of variance. Subsequent analyses on data within doses were 2 (pregnancy) × 4 (time) analysis of variance. For all analyses, the time factor was a repeated measure.
17. The data were subjected to analyses of variance across time within each dose, and the groups receiving different drug doses were compared to saline controls via Dunnett's tests. Probabilities ≦ .05 were accepted as significant differences.
18. J. E. King and R. F. Becker, *Am. J. Obstet. Gynecol.* **86**, 856 (1963); ———, R. T. James, *ibid.*, p. 869; J. E. King, *ibid.* **89**, 1019 (1964).
19. Blood was collected into 75 μl capillary tubes from the orbital sinus. Animals were decapitated within 60 seconds and brains removed. Blood samples were centrifuged for 6 minutes and plasma was collected. Samples were stored at −65°C until assayed. Plasma concentrations of phenobarbital were determined by adding 40 μl of plasma to a 15-ml culture tube containing 120 mg of KCP. Acetonitrile (2.5 ml) containing the internal standard (Primidone) was added and the tube was then vortexed. Hexane (2.5 ml) was added, the tube vortexed again and then spun at top speed for 5 minutes in a Dynac centrifuge (Clay-Adams). A portion (2.0 ml) of the acetonitrile layer was placed into another conical centrifuge tube and evaporated to dryness under nitrogen. The residue was taken up in 200 μl of the HPLC (high-performance liquid chromatography) mobile phase and 40 μl of the solution was injected onto the HPLC column. Brain concentrations of the drug were determined by homogenizing the brain in 10 volumes of 0.1*M* $NaHPO_4$ buffer (*p*H 7.45) with a Brinkmann Polytron. The homogenate was then centrifuged (Sorvall RC-5) at 27,000*g* for 10 minutes. A portion (2 ml) of the supernatant was added to a tube containing 1.2 g of KCl. The phenobarbital was then extracted with 4.0 ml of acetonitrile and 4.0 ml of hexane as described for plasma. A portion (3 ml) of the acetonitrile layer was evaporated and the residue taken up in 200 μl of the HPLC mobile phase. Of this, 40 μl was injected onto the HPLC column. The extracted phenobarbital samples were chromatographed on a Waters μBondapak C-18 column with the use of a Waters 6000-A pump, Waters injector, and an LKB Uvicord S monitor with a 206-nm lamp. The mobile phase was 25 percent methanol and 75 percent KH_2PO_4 buffer, *p*H 4.4 (0.15 m*M*). The flow rate was 2.5 ml/min.
20. In spite of lack of statistical support for a pregnancy × time interaction, mean brain concentrations appeared to peak later in pregnant than nonpregnant animals injected with the 80 mg/kg dose. Comparisons of means across time periods within each pregnancy condition by

Newman-Keuls tests established that brain concentrations at 30 minutes were significantly higher than those at 15 minutes for both pregnant and nonpregnant mice. A further increase was observed at 60 minutes for pregnant but not nonpregnant animals, indicating that peak brain concentrations may have occurred later for pregnant mice. The maximum concentration obtained, however, was similar for both groups.

21. A. Blackham and P. S. J. Spender, *Br. J. Pharmacol.* **37**, 129 (1969); A. Blackham, *J. Pharm. Pharmacol.* **22**, 304 (1970); C. L. Rumke and J. Noordhoek, *Eur. J. Pharmacol.* **6**, 163 (1969).
22. S. Barlow, P. J. Morrison, F. M. Sullivan, *J. Endocrinol.* **60**, 473 (1974); P. R. McElhatton and F. M. Sullivan, *Teratology* **12**, 97 (1975).
23. H. Nishigori and M. Iwatsuru, *Life Sci.* **30**, 433 (1982).

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Synaptic Activity Mediates Death of Hypoxic Neurons

Abstract. *Cultured hippocampal neurons, when exposed to cyanide or an anoxic atmosphere in the early stages of differentiation, were not visibly affected. However, neurons in mature cultures died when exposed to cyanide or anoxia. Cell death could be prevented by treatment with magnesium, which eliminates synaptic activity. These observations suggest that damage in hypoxic neurons is mediated by synaptic activity.*

A number of clinical disorders, including stroke, perinatal asphyxia, and shock, result in an insufficient supply of oxygen to the brain. Lack of oxygen leads to a cessation of cerebral function that becomes irreversible if the oxygen supply is not replenished. The pathological concomitant of irreversible cerebral dysfunction is extensive death of neurons. The precise mechanism responsible for the death of hypoxic neurons is unknown (*1*). Most studies of brain hypoxia in intact animals are difficult to interpret because other variables, such as hypotension, hypercarbia, and acidosis, contaminate the experimental design. The problem of studying hypoxia as an isolated variable can be partly circumvented by using cultured neurons. Cultures of central neurons undergo structural changes within 15 minutes of exposure to low oxygen tension (*2*).

We used cultures of rat hippocampus to define more precisely the factors responsible for the death of hypoxic neurons. The cultures were grown from dissociated hippocampal neurons obtained from fetal rats in the 18th day of gestation. A suspension of 1.5×10^5 cells was plated into poly-L-lysine–coated culture dishes (diameter, 35 mm) containing 1.5 ml of synthetic medium supplemented with 10 percent human serum (*3*). Over the first 2 weeks in vitro these cells form an extensive network of processes and develop spontaneous synaptic activity (*4*, *5*). Intracellular recording has demonstrated physiological and pharmacological properties similar to those of hippocampal neurons in other preparations in vivo and in vitro (*5*).

Addition of 1 m*M* NaCN (to duplicate the effects of hypoxia) had no effect on cultures less than 2 days old. Cells that were added to media containing NaCN attached normally to the culture dish and began extending processes. Cyanide has been reported to decrease neuronal adhesion to polylysine at higher concentrations, but did not affect adhesion in this system (*6*). Cell loss was the same in control and NaCN-containing cultures (Fig. 1).

In contrast, NaCN had marked effects on cultures older than 2 weeks. Within 1 hour the neurons became swollen and vacuolated, and by 4 hours they had started to disintegrate. After 1 day they had been replaced by debris (Fig. 2A).

Similar results were obtained when

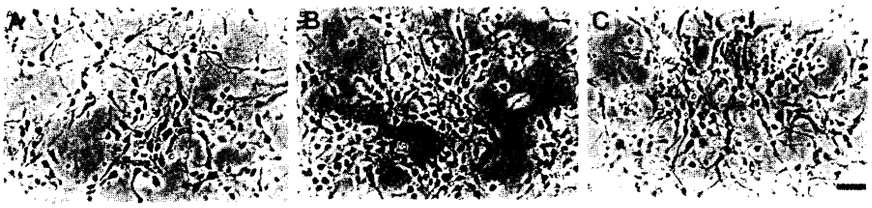

Fig. 1. (A) Phase-contrast photomicrograph of control hippocampal cells after 2 days in vitro. (B) Two-day-old cells that were plated and grown in medium containing 1 m*M* NaCN. (C) Two-day-old cells that were placed in the anoxic atmosphere 5 hours after plating. They remained in the anoxic environment for the next 43 hours. Scale bar, 50 μm.

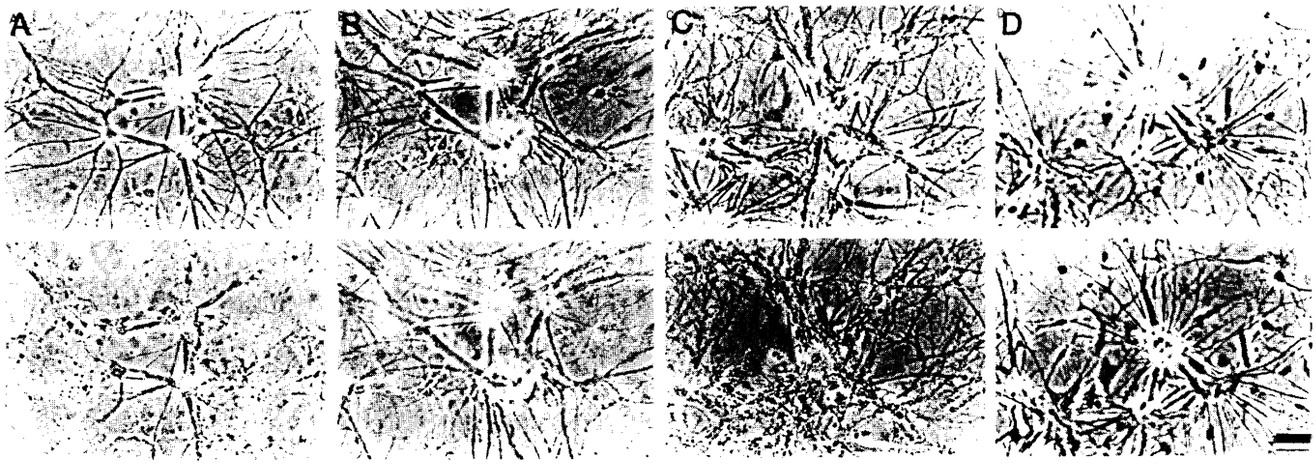

Fig. 2. (A) Phase-contrast photomicrographs of hippocampal neurons after 23 days in vitro. The same field is shown before (top) and after (bottom) 21 hours of exposure to 1 m*M* NaCN. The neurons died and were replaced by debris. (B) Hippocampal neurons (23 days in vitro) before and after 21 hours of exposure to 1 m*M* NaCN, in which the culture was first treated with 10 m*M* $MgCl_2$. There is virtually no change in the neurons. (C) Neurons (18 days in vitro) before and after exposure to the anoxic atmosphere. As in (A), the neurons have died, leaving only debris. (D) Neurons (18 days in vitro) before and after exposure to the anoxic atmosphere for 14 hours, in which the culture was first treated with 10 m*M* $MgCl_2$. The neurons are still intact. Scale bar, 50 μm.