restial environments: *H. pulcher, H. lateristrigatus, C. inguinalis, Ptychadena mascareniensis*, and *Phrynobatrachus natalensis*. Extracts from these five species and from the terrestrial *Phyllobates bicolor* all inhibited uptake of rubidium ions into red blood cells to an extent roughly consonant with their potency in inhibiting binding of ouabain to Na^+,K^+-ATPase. Thus, at least in these frogs, the active compounds not only compete for ouabain-binding sites but also inhibit Na^+,K^+-ATPase.

In summary, the skin of many amphibians contains inhibitory compounds that interact with the ouabain-binding site of Na^+,K^+-ATPase. The presence of such compounds appears highly variable except in the family Bufonidae, whose species contain high titers of compounds that inhibit ouabain binding and Na^+,K^+-ATPase activity. In skin secretions, these compounds probably serve in defense against predation. In *Bufo* and *Atelopus* (*7*), the active compounds are bufodienolides, whereas *Dendrophryniscus minutus* contains high titers of polar compounds that, based on the absence of a significant ultraviolet chromophore, do not appear to be bufodienolides. It would be inappropriate to draw taxonomic conclusions concerning intergeneric relations in the Bufonidae until the structures of the active polar compounds in *Dendrophryniscus* and *Melanophryniscus* are clarified. But it is of interest that extremely high levels of Na^+,K^+-ATPase inhibitors in the skin may be characteristic of the family and not limited to *Bufo*. The structures of inhibitory compounds present at low titers in species of other families are also unknown. They may represent physiological regulators for Na^+,K^+-ATPase in amphibian skin.

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6. Skin from juvenile *B. marinus* (length, 2 to 3 cm) had levels of active substances only somewhat lower than those from adults. Skins from 80 tadpoles (Santa Barbara, upper Orinoco River, Venezuela) contained about 50 ouabain equivalents per milligram.
7. Methanol extracts from *A. ignescens* were analyzed further by high-pressure liquid chromatography (Zorbax ODS column with a linear gradient programmed from 50 to 80 percent methanol in H_2O over 30 minutes). The results suggested the presence of telocinobufagin and bufotalin as major constituents and marinobufagin, cinobufagin, bufalin, arenobufagin, and two unidentified bufodienolides as minor constituents. Standard compounds eluted in the following order: gamabufotalin, arenobufagin, hellebrigenin, bufotalin, telocinbufagin, bufalin, cinobufagin, resibufogenin, and marinobufagin. The two unidentified bufodienolides in the *Atelopus* extract eluted between hellebrigenin and bufotalin.
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9. Human red cells were prepared as described in (*4*) and resuspended at a hematocrit level of 15 percent in buffer containing 150 m*M* NaCl, 10 m*M* tris, 10 m*M* dextrose, and 2 m*M* RbCl at a *p*H of 7.4. Ouabain or skin extracts (25 μl) were added to 200 μl of cell suspensions and incubated at 37°C for 60 minutes. The cells were washed and reconstituted with 200 μl of buffer. After addition of 0.4 μCi of $^{86}Rb^+$ (3.1 mCi/mg), the cells were incubated for 70 minutes at 37°C and then washed three times in buffer containing 150 m*M* NaCl. The final pellet was treated with 100 μl of 10 percent perchloric acid. After sedimentation of the pellet, the tubes were inverted into scintillation vials containing 5 ml of 150 m*M* NaCl. Influx of $^{86}Rb^+$ was determined by subtraction of the values obtained at zero time.
10. Low titers of compounds that inhibit ouabain binding were detected in methanol extracts from the skins of the North American salamanders *Notophthalmus viridescens* and *Pseudotriton ruber*.
11. The possible significance of environmental factors is suggested from preliminary results with *Dendrobates auratus*: frogs obtained in the dry season on Isla Taboga, Panama, contained 0.08 ouabain equivalent per milligram of skin (Table 1), whereas skin from frogs collected in the wet season contained only 0.01 equivalent per milligram. Eight frogs maintained for 4 months in a terrarium had 0.5 equivalent per milligram of skin. After 5 hours in isotonic saline, levels of ouabain equivalents in two terrarium frogs had increased by >50 percent.
12. A ouabain equivalent is equal in activity to that amount of ouabain (4 ng) which inhibits binding of [^{3}H]ouabain to Na^+,K^+-ATPase by 50 percent.
13. Human red blood cells were prepared and incubated with $10^{-9}M$ [^{3}H]ouabain (8 Ci/mmole) for 60 minutes at 37°C, either alone or in the presence of increasing concentrations of nonradioactive ouabain ($10^{-8} \times 10^{-5}$ to $3 \times 10^{-5}M$) or portions of methanol or aqueous extracts equivalent to 0.001 to 20 mg of skin. Centrifugation, washing, and scintillation spectroscopy were carried out as described in (*4*), and ouabain equivalents were calculated through comparison with the ouabain displacement curve. (Maceration of skins twice each time with 5 ml of methanol per gram of skin had yielded the methanol extracts. In some cases, skins were then macerated twice with water to yield an aqueous extract.) When partition between aqueous methanol and chloroform was obtained, the chloroform phase was evaporated and redissolved in methanol.
14. We gratefully acknowledge the assistance of D. McIsaac and P. Nikada. This research was supported in part by a grant from the American Heart Association. Most of the samples from Peru were obtained during the Alpha Helix Amazon Expedition (April 1977), which was supported by NSF grant 7605271. Most of the other fieldwork was financed by the Lincoln Ellsworth Fund of the American Museum and by a grant to the museum from the Camille and Henry Dreyfus Foundation. The work in Brazil was done in collaboration with P. E. Vanzolini of the Museu de Zoologia, Universidade de São Paulo. The suggestion to study *Bufo* tadpoles was made by S. Rand, Smithsonian Tropical Research Institute, Panama. Bufodienolides were provided by K. Meyer of the University of Basel, Basel, Switzerland.

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L-Ornithine Decarboxylase: An Essential Role in Early Mammalian Embryogenesis

Abstract. *The highly selective, enzyme-activated, irreversible inhibitor of* L-*ornithine decarboxylase,* DL-α-*difluoromethylornithine, suppresses the increase in uterine* L-*ornithine decarboxylase activity associated with early embryogenesis in the mouse and arrests embryonic development at that stage. Contragestational effects were confirmed in the rat and rabbit. An increase in* L-*ornithine decarboxylase activity that leads to a rapid increase in putrescine concentration appears to be essential during a critical period after implantation for continued mammalian embryonal growth.*

Decarboxylation of L-ornithine by L-ornithine decarboxylase (ODC) (E.C. 4.1.1.17) leads to the formation of putrescine and is the initial step in the biosynthesis of the polyamines spermidine and spermine (*1, 2*). Although basal activity of ODC is low in most tissues, marked increases are characteristically associated with rapid tissue growth (*3*) and particularly with mammalian and nonmammalian embryogenesis (*4*).

Although such observations imply a functional role for ODC and the polyamines in the regulation of growth processes, direct proof of this has been difficult to obtain, principally because of the lack of tools with which to manipulate selectively the polyamine biosynthetic pathway (*3*). A potent and selective inhibitor of ODC that works through the mechanism of substrate-induced, irreversible inhibition (*5*) was recently developed in our laboratories (*6*). This compound, DL-α-difluoromethylornithine (α-DFMO) (RMI 71782), proved an effective inhibitor of ODC in vivo (*7, 8*). We now report that α-DFMO arrests embryonic development in three mammalian species. The results provide direct evidence that the changes in ODC associated with the early stages of mammalian embryogenesis are essential to the process and not merely an incidental, noncausal event.

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Mice of strain CDA, HAM-ICR were killed on the appropriate day of pregnancy to provide tissues for determination of ODC and *S*-adenosyl-L-methionine decarboxylase (SAM-DC) (E.C. 4.1.1.50) activities and polyamine concentrations by methods previously described (*8*). Figure 1A reveals that ODC activity in the whole uterus was low during the first 5 days of gestation but increased sharply between days 6 and 8 to reach a peak on day 8 of gestation. The activity declined significantly by days 9 and 10. Similar changes occurred in the concentration of putrescine (Fig. 1B). The activity of SAM-DC increased slowly over the same time period and reached peak activity on day 9 of gestation (Fig. 1C). Spermidine concentrations increased significantly during this time (Fig. 1D) and the uterine sper-

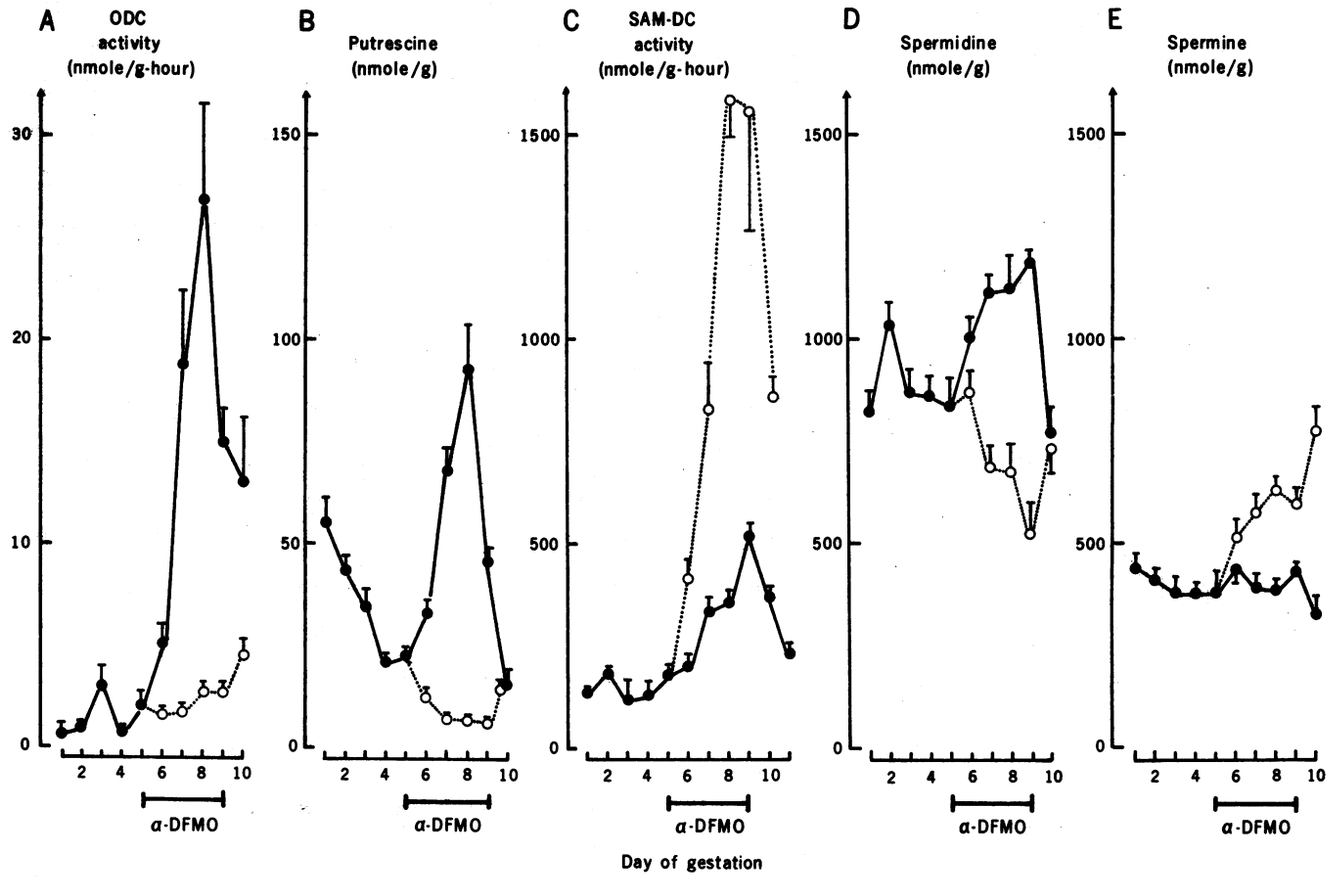

Fig. 1. Changes in polyamine biosynthetic enzyme activities and polyamine concentrations in mouse uterus during early gestation. Effects of treatment with α-DFMO. Symbols: ●, untreated control animals; ○, animals receiving α-DFMO (2 percent in the drinking water) during days 5 to 8 of gestation (mean daily drug intake, 3000 mg/kg). Values are means ± standard error of four to ten determinations at each time point.

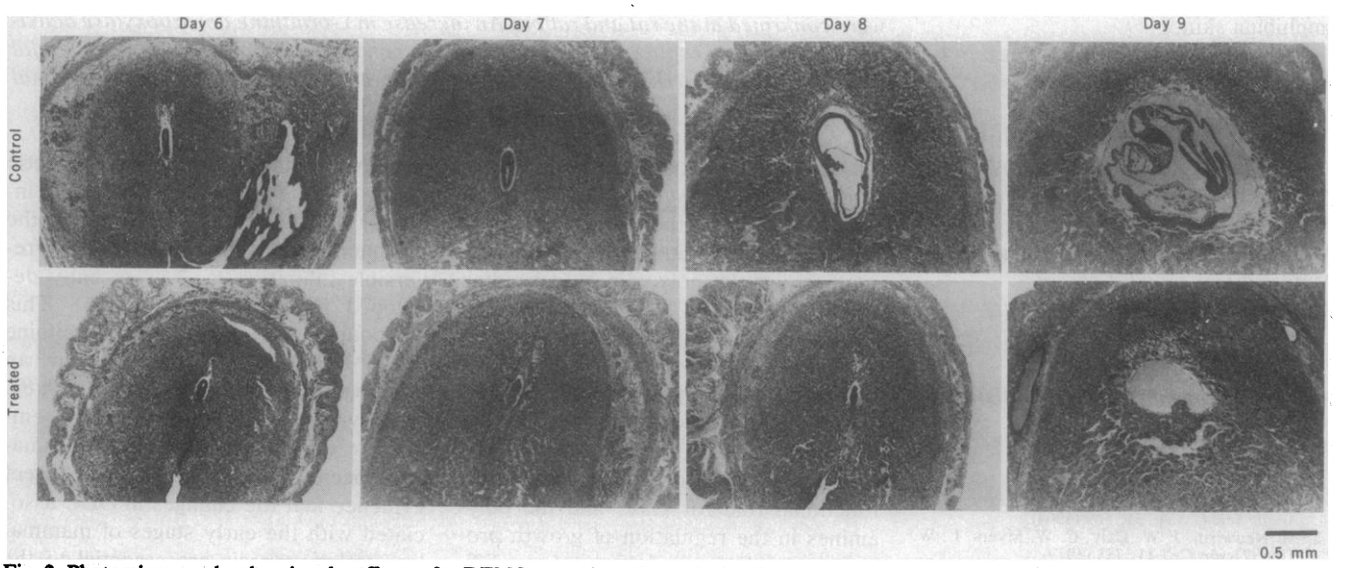

Fig. 2. Photomicrographs showing the effects of α-DFMO on early embryonic development in the mouse. An apparently normal decidual reaction takes place during treatment with α-DFMO. However, embryonic development fails to progress beyond a stage typical of day 7 or 8 of normal gestation. The animals received α-DFMO (2 percent in the drinking water) during days 5 to 8 of gestation (mean daily drug intake, 3000 mg/kg).

mine levels were unchanged (Fig. 1E). Implantation in the mouse takes place on days 4 to 5 of gestation. The changes in polyamine metabolism thus correspond to the initial stages of embryogenesis after successful implantation.

The biochemical data suggested days 5 to 8 as the obvious point during early murine gestation when intervention with an inhibitor of ODC might produce maximum biological effects. Mice were treated with α-DFMO during this period by including it in the drinking water at concentrations of 0.125, 0.5, or 2 percent resulting in mean daily intakes (for a 40-g mouse) of 259, 988, and 3070 mg per kilogram, respectively.

To assess the biological consequences of such treatment, we killed both treated and control mice 24 hours before the expected date of parturition on day 18 of gestation and examined the uteri and their contents. Treatment with the 0.125 percent solution of α-DFMO had only a minimum effect on gestation (Table 1). In contrast, treatment with 0.5 percent α-DFMO produced several significant effects. First, fewer mated mice were impregnated in comparison to the control group. Second, each gravid female contained, on average, only two viable fetuses and showed a compensating increase in the number of resorption nodules. Finally, both the fetal and placental weights were reduced compared to those in the control group. The results from the animals that received the 2 percent solution of α-DFMO were unequivocal; none of the 19 mice that were proved to have mated showed any signs of pregnancy when autopsied on day 18 of gestation (Table 1).

Biochemical analysis of the uteri showed that in animals treated with 2 percent α-DFMO during days 5 to 8 of gestation the increases in uterine ODC activity and in putrescine and spermidine concentrations seen during normal gestation were abolished (Fig. 1, A, B, and D). In contrast, SAM-DC activity was markedly increased after treatment with α-DFMO (Fig. 1C). This probably accounts for the resistance to depletion of the uterine spermidine concentrations (Fig. 1D) and for the small but significant increase in the concentration of spermine (Fig. 1E).

The photomicrographs (*9*) in Fig. 2 show that in animals treated with α-DFMO decidualization takes place normally after implantation, but that embryonic development fails to progress beyond a stage typical of days 6 to 7 of normal gestation, the time when uterine ODC begins to increase in control animals (Fig. 1A). Subsequently, the arrested embryo and the surrounding decidual tissue showed increasing signs of rejection, the remains being resorbed or lost from the uterus between days 16 and 18 of gestation. The uterus at 18 days was histologically normal; it was also functionally normal, since animals so treated produced standard litters when mated at the first estrus after treatment.

We also examined the effects of α-DFMO on female Sprague-Dawley rats and Polish rabbits. These animals were time-mated and treated with α-DFMO during the period immediately following implantation corresponding to that during which, in the mouse, embryonic development was susceptible to inhibition. Thus, rats were offered a 2 percent solution of α-DFMO (providing a mean daily intake of 2023 mg/kg) as the sole drinking fluid on days 5 to 9 of gestation; rabbits received a 3 percent solution between days 6 and 10 of gestation, resulting in a mean daily intake of 1799 mg/kg. Animals were killed 24 hours before parturition (day 19 in rats; day 30 in rabbits) and the uteri and their contents were examined. The data (Table 2) confirm for the rat and the rabbit the observations made in the mouse. Thus implantation appeared to take place normally, but there was an arrest of early embryonic development since no developed fetuses were found in the uteri of either species.

The marked increases in ODC activity and polyamine concentrations associated with early murine embryogenesis (Fig. 1) confirm for the mouse the obser-

Table 1. Effects of α-DFMO on gestation in mice. Autopsies were performed on the treated and control mice 24 hours before parturition on day 18 of gestation. In all experiments, 311 mice with vaginal plugs yielded 274 pregnancies (88 percent). The difference between this expected figure and the figures obtained after treatment with 0.5 or 2.0 percent α-DFMO reflects the loss or reabsorption of the nodules prior to day 18 of gestation. For this reason the data are expressed both in terms of the number of mice known to have mated and the number found to be gravid at autopsy. The values presented are means (± standard error) of the number of individual determinations given in parentheses.

Item	Concentration (percentage) of α-DFMO in drinking water			
	0.0	0.125	0.5	2.0
Number of mice				
Mated	13	7	13	19
Gravid at autopsy	10 (77 %)	6 (86 %)	7 (54 %)	0 (0 %)
Viable fetuses				
Per mated mouse	7.5 ± 1.5 (13)	6.1 ± 1.5 (7)	1.1 ± 0.6* (13)	0
Per gravid mouse	9.8 ± 1.3 (10)	7.2 ± 1.4 (6)	2.0 ± 1.0* (7)	
Nonviable fetuses				
Per mated mouse	0.4 ± 0.2 (13)	0.1 ± 0.1 (7)	0.1 ± 0.1 (13)	0
Per gravid mouse	0.5 ± 0.3 (10)	0.2 ± 0.2 (6)	0.1 ± 0.1 (7)	
Resorption nodules				
Per mated mouse	2.3 ± 0.6 (13)	3.0 ± 0.9 (7)	5.6 ± 1.7 (13)	0
Per gravid mouse	3.0 ± 0.6 (10)	3.5 ± 0.9 (6)	10.4 ± 1.5* (7)	
Mean weight of Viable fetuses (mg)	1096 ± 19 (98)	921 ± 22* (43)	656 ± 20* (14)	
Placentas of viable fetuses (mg)	122 ± 2 (98)	127 ± 3 (43)	101 ± 5† (14)	

*$P < .001$. †$P < .01$, compared to controls.

Table 2. Effects of α-DFMO on gestation in rats and rabbits. The rats received 2 percent α-DFMO in their drinking water on days 5 to 9, and the rabbits 3 percent α-DFMO on days 6 to 10. The values presented are means (± standard error) of the number of individual determinations given in parentheses.

Item	Rats		Rabbits	
	Water	α-DFMO	Water	α-DFMO
Number of animals				
Mated	14	14	4	5
Impregnated	13 (93 %)	12 (86 %)	4 (100 %)	5 (100 %)
Per gravid female, number of				
Implantations*	14.1 ± 0.3 (13)	14.4 ± 0.5 (12)	5.0 ± 0.4 (4)	5.4 ± 0.7 (5)
Viable fetuses	13.1 ± 0.4 (13)	0	5.0 ± 0.4 (4)	0
Nonviable fetuses	0.1 ± 0.1 (13)	0	0	0
Resorption nodules	0.9 ± 0.3 (13)	12.7 ± 1.3† (12)	0	5.4 ± 0.7 (5)

*Total of viable and nonviable fetuses plus resorption nodules. The figures for rats were obtained from the number of maternal placentas. The difference between number of implantations and number of resorption nodules per pregnant female rat reflects the number of nodules lost or resorbed by day 19. †$P < .001$, compared to controls.

vations made previously for the early development of the chick, rat, and a number of amphibian and invertebrate species (*4, 10, 11*). These increases precede, or occur simultaneously with, increases in DNA, RNA, and protein synthesis (*2, 3*), and strongly imply a fundamental role for ODC and the polyamines in the phase of rapid growth associated with early embryonic development. Our data in this report support this conclusion in that they show that α-DFMO, an irreversible inhibitor of ODC, suppresses the increases in ODC and the polyamines associated with early murine gestation and arrests embryonic development at that point. However, it has recently been emphasized that the validity of such evidence depends entirely on proof of the specificity of the inhibitory drug for the particular metabolic pathway involved (*12*). On mechanistic grounds specificity would be predicted since α-DFMO works through the principle of substrate-induced inhibition (*5*). Thus, α-DFMO does not directly inhibit ODC irreversibly. Rather it is accepted by the enzyme as a substrate (*6*) and decarboxylated to yield a highly reactive intermediate which alkylates the enzyme and inactivates it irreversibly. In practice, the pattern of polyamine biochemical changes produced by α-DFMO is entirely consistent with selective inhibition of ODC. Further, α-DFMO does not inhibit other 1-carboxylyases such as glutamic acid decarboxylase, histidine decarboxylase, or aromatic L-amino acid decarboxylase (*6*), or SAM-DC (Fig. 1). Finally, the compound has no acute pharmacological activity and is essentially nontoxic, there being no untoward effects following a single oral dose of 5000 mg/kg. There is, therefore, no apparent explanation for the contragestational effects of α-DFMO other than inhibition of ODC. The close temporal correlation between inhibition of the peak rise in enzyme activity and arrest of embryonic development further supports this conclusion.

Barkai and Kraicer (*13*) found that substantial increases in ODC were associated exclusively with the maternal decidual reaction in the rat. We have confirmed this observation for the mouse (*14*), but we do not know whether the decidualizing tissue or the embryo itself is the site where the inhibition of ODC is functionally important. The histological evidence (Fig. 2) suggests that the decidual reaction takes place normally despite treatment with α-DFMO. Further, in recent experiments, contragestational effects have been obtained by administration of α-DFMO on day 8 of gestation only, at which time decidualization is established (*15*). The embryo, therefore, seems to be the functionally important site for inhibition of ODC. Decidualization may represent an example of a growth process which is associated with, but not dependent on, an increase in ODC activity.

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Phenobarbital Exposure in Utero: Alterations in Female Reproductive Function in Rats

Abstract. *Phenobarbital administration to pregnant rats from day 12 to day 19 of gestation suppressed body weight gain and produced significant effects on reproductive function in their offspring. These effects included delays in the onset of puberty, disorders in the estrous cycle, and infertility. Moreover, the animals exposed to phenobarbital in utero showed altered concentrations of sex steroids, gonadotropic hormones, and estrogen receptors. These findings suggest that phenobarbital treatment during prenatal development can produce permanent alterations in sexual maturation.*

Psychotropic agents such as tranquilizers and sedatives represent the major class of drugs prescribed to the pregnant woman (*1, 2*). Traditionally, investigators have studied the effects on fetuses of exposure to drugs and chemicals during organogenesis, since such exposure can cause malformations. Prenatal exposure to drugs, however, may also produce functional disturbances. We have used rats to study the long-term effects on reproductive function of exposure to phenobarbital in utero. This drug, which is widely used during human pregnancy, is known to block ovulation (*3–5*) in the adult rat, and treatment of neonatal rats with phenobarbital can alter adult sexual behavior (*6*). Our results show that phenobarbital has profound effects on reproductive function in female rats exposed to the drug in utero. These effects include a delay in the onset of puberty, disorders in the estrous cycle, and infertility.

Phenobarbital was administered to pregnant rats (Sprague-Dawley, CD strain) in a single subcutaneous injection in the morning from day 12 to day 19 of pregnancy. Each rat received 40 mg of the drug per kilogram of body weight per day. Control pregnant rats received injections of saline. Day 0 of gestation was the day on which a vaginal smear positive for sperm was first obtained. Six pregnant animals were assigned to each treatment group in every experiment. At birth the number of offspring in each litter was randomly adjusted to 8 or 10, with equal numbers of each sex in every group. Animals were weighed only once every week and weaned at 21 days of age. Food and water were constantly available. The onset of puberty was determined from the time of vaginal open-

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