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Influence of Diphenylhydantoin on Spontaneous Release of Ovulating Hormone in the Adult Rat. (30354)

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A number of pharmacological agents are capable of blocking spontaneous ovulation in the rat when given prior to the "critical period"(1). With the rats used in this study the "critical period," that is, the period of neurohumoral stimulation of the anterior pituitary gland for the release of ovulating hormone, occurs between 14:00 and 16:00 on the afternoon of proestrus(2). Atropine, pentobarbital (Nembutal), chlorpromazine, morphine or reserpine blockade of spontaneous ovulation in the rat can be overcome by applying an "electrolytic" stimulus to the medial preoptic area(3,4, unpublished). This information suggests that these neuropharmacological agents exert their inhibitory effect on gonadotropin release primarily by way of their influence on the central nervous system. The present study is concerned with the influence of diphenylhydantoin (DPH), an anticonvulsant, on spontaneous ovulation in the rat. Experimental evidence has been presented which indicates that diphenylhydantoin interferes with gonadotropin release in the rabbit (see *Discussion*) and with reflex adrenocorticotropin release in the rat(5) and man(6). The possible mechanisms by which diphenylhydantoin alters the central nervous system to exert its anticonvulsant activity as well as its peripheral sites of action have been recently reviewed(7,8).

Material and methods. Adult albino rats from an inbred Osborne-Mendel derivative strain weighing between 180 and 255 g were used. The animals were maintained on Purina lab chow and water *ad libitum*. With the animals existing under the regimen of 14 hours of light and 10 hours of darkness, the "critical period" occurred between 14:00 and 16:00, that is, 8 to 10 hours following the onset of light(1). The experimental animals were used on the day of proestrus with 2 previous 4-day cycles being a prerequisite for their selection.

Diphenylhydantoin (DPH) was dissolved in a solvent consisting of 40% propylene glycol and 10% alcohol in water and adjusted to pH 12 with sodium hydroxide. DPH was dissolved in the solvent at a concentration of 50 mg per cc. Single intraperitoneal injections were made either just prior to or about 2½ hours before the "critical period."

A stock solution of NIH-LH-B_1† (bovine LH) was prepared with physiological saline and stored in a frozen condition until needed. Appropriate dilutions were made with physiological saline and the hormone was injected subcutaneously. A standard dose of bovine LH (8 μg/100 g body weight) is capable of consistently inducing the full ovulatory re-

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sponse in pentobarbital-blocked, proestrous rats (Everett & Redmond, unpublished observations). Two animals were tested in the present series to confirm the activity of this dose and both animals shed full sets of ova.

"Electrolytic" stimulation of the brain was carried out according to the techniques extensively described by Everett(9). Briefly, a direct current of 10 μamp/60 seconds was applied through a stainless steel concentric bipolar electrode (SCE), the current being monitored through a cathode ray oscilloscope. Unilateral irritative foci of iron were set up following this type of stimulation(10). The electrode was lowered from Bregma a vertical distance of 7.5 mm and the tip of the electrode rested approximately 0.5 mm lateral to the midline in the medial preoptic area. With the coordinate system used in this laboratory(4) the frontal plane at Bregma passes in front of the anterior commissure.

Vaginal smears were taken immediately before autopsy the morning following treatment. Subsequently, the oviducts were removed and ova counts were carried out with the aid of a binocular microscope(11). In this series of experiments full ovulation is represented as the presence of 7 or more ova in the oviducts on the morning of autopsy. Partial ovulation is represented as the presence of less than 7 ova. The ovaries were removed and fixed in Bouin's solution in all cases in which ovulation was entirely or partially absent. The ovaries were cut at 10 μ and stained with hematoxylin, acid fuchsin, orange G and aniline blue. The animals were perfused with physiological saline followed by 10% formalin in saline and several days later the brains were removed and placed in a postfixative(12). The brains were embedded in paraffin, sectioned at 20 μ and stained with Luxol Fast Blue and neutral red.

Results. Influence of dose and time of diphenylhydantoin injection on spontaneous ovulation. When DPH was administered just prior to the "critical period" ovulation was inhibited in many of the animals (Table I). Only the lowest dose (50 mg/kg) failed to interfere with spontaneous ovulation when given at that time. Treatment with DPH prevented spontaneous ovulation to a greater degree when given 2½ hours before the "critical period" as compared with treatment immediately before it (Table I).

TABLE I. Effect of Dose and Time of Administration of Diphenylhydantoin on Spontaneous Ovulation.

DPH (mg/kg)	No. blocked	No. partially blocked	No. full ovulation
(13:45-14:00)*			
50	—	—	2
100	2	—	2
150	4	1	3
200	2	—	1
(11:30-12:00)			
DPH solvent†	—	—	3
100	10	2	5
125	3	2	1
175	7	—	—
200	4	—	1

* Time of DPH injection.
† DPH solvent (volume = 0.8 cc) injected intraperitoneally.

Three control animals possessed full sets of ova, full vaginal cornifications, and contracted uteri on the morning following intraperitoneal injection of the solvent for DPH. These characteristic changes are observed on the morning of estrus in the untreated rat. On the other hand, animals not ovulating following DPH treatment had retarded smears, that is, late proestrous smears, and the uterine lumens were distended with fluid.

Neurotoxicity was evident only following DPH doses larger than 100 mg/kg. Slight ataxia was noted during the "critical period" after injection of either 125 mg/kg or 150 mg/kg of DPH. No signs of ataxia were observed the next morning. However, when 175 mg/kg or 200 mg/kg of DPH were injected on the day of proestrus marked ataxia followed and persisted until the morning of autopsy.

Influence of exogenous gonadotropin or hypothalamic stimulation on ovulation in the DPH-blocked rat. When the lower dose of DPH (100 mg/kg) was given, full ovulation in all animals followed either administration of exogenous LH or "electrolytic" stimulation of the medial preoptic area (Table II). The mere placement of an electrode into the medial preoptic area failed to alter the inhibitory effect of DPH on spontaneous ovulation. This information suggests that DPH,

TABLE II. Results of Exogenous Gonadotropin or Hypothalamic Stimulation on Ovulation in DPH-Blocked Rat.

Treatment*	No. blocked	No. partially blocked	No. full ovulation
DPH_{100}†	10	2	5
DPH_{100} + LH_8‡	—	—	5
DPH_{100} + MPOA-stim.§	—	—	4
DPH_{100} + E-placement-MPOA	3	1	—
DPH_{175}	7	—	—
DPH_{175} + LH_{12}	—	2	2
DPH_{175} + MPOA-stim.	2	—	3
DPH_{175} + E-placement-MPOA	1	—	—

* DPH injected between 11:30-12:00; LH injected between 15:45-16:15.
† Dose in mg/kg.
‡ Dose in μg/100 g body wt.
§ 10 μa/60 sec, direct current, SCE.

at seemingly nontoxic doses, interferes with spontaneous ovulation by altering the central nervous system's control of gonadotropin secretion.

Peripheral interference with the ovulatory response occurred after either exogenous LH or preoptic stimulation when a larger dose of DPH was used. Following 175 mg/kg of DPH, the administration of exogenous LH (12 μg/100 g body wt) produced complete ovulation in 2 animals and partial ovulation in 2 others. The ovaries of the animals demonstrating partial ovulation possessed marked preovulatory changes and a few freshly ruptured follicles. "Electrolytic" stimulation of the medial preoptic area of animals pretreated with DPH (175 mg/kg) produced full ovulation in 3 animals and negative results in 2 animals. The ovaries of the latter showed marked preovulatory changes but no ovulated follicles.

Discussion. Absence of spontaneous ovulation was observed in the majority of animals receiving diphenylhydantoin before the "critical period." Blockade of the ovulatory response with the lower dose of DPH (100 mg/kg) was overcome with either exogenous LH or preoptic stimulation. Apparently the drug exerts its effect on the nervous system exclusive of the preoptic region of the hypothalamus. Preoptic stimulation has also been shown to overcome the blockade of spontaneous ovulation in the rat by atropine, pentobarbital, chlorpromazine, morphine or reserpine(3,4, unpublished). Even in the presence of larger, neurotoxic doses of DPH, preoptic stimulation induces the release of endogenous LH. Although a reduced number of animals showed full sets of ova following preoptic stimulation a similar response occurred after injection of a known ovulating dose of LH. Peripheral interference with hormonal transport or action appears evident in these latter cases.

Studies carried out in the rabbit by Christian and McDowell (unpublished, personal communication from Dr. C. D. Christian) revealed that diphenylhydantoin (20 mg/kg-I.V.-3 consecutive days) prevented ovulation following mating in 4 out of 7 animals. DPH (40 mg/kg-I.M.) administered for 10 consecutive days prevented ovulation in 12 out of 15 rabbits following electrical stimulation of the basal tuberal-posterior median eminence region of the hypothalamus. Eight of 10 control animals ovulated following similar stimulation. When more intense electrical stimulation was applied to the hypothalamus of the DPH-treated rabbits, ovulation occurred in all 3 animals tested. The authors concluded that diphenylhydantoin interfered with the release of luteinizing hormone by way of its action on the nervous system, and that this blockade could be overcome only by increased amounts of electrical stimulation of the hypothalamus. In view of the present studies in rats, peripheral interference with the ovulatory response following DPH in the rabbit remains to be ruled out before final conclusions can be drawn.

Summary. It may be concluded from the present studies that diphenylhydantoin interferes with the neural stimulus that is responsible for the discharge of the hypophysiotrophic principles necessary for the release of ovulating hormone from the adenohypophysis. Artificial stimulation of the medial preoptic area in the DPH-blocked rat is capable of provoking the release of ovulating hormone. Subsequent peripheral interference with the ovulatory process, sites undeter-

mined, occurs only with the use of the higher dose of diphenylhydantoin.

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Reactions Between Human Serum Albumin and Several Hemoglobins.* (30355)

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Components of human serum form both soluble and insoluble complexes with hemoglobin. Soluble complex formation has been the most studied, and has been demonstrated between hemoglobin and the haptoglobins (1), hemophylline(2), and between the heme group of hemoglobin and albumin(3).

Recently, the ability of human serum to form insoluble complexes with hemoglobin in agar gels has been described(4-6). It has been suggested that the serum participant in this reaction is albumin(4,6) or an α_2 globulin(5). This report will present a technique for quantification of this precipitin reaction, and will offer evidence that albumin is the serum reactant. Reactions involving several different natural hemoglobins as well as several chemically altered hemoglobins will be described.

Materials and methods. Fresh hemolysates (7) as well as 2× crystallized hemoglobins (Pentex, Inc., Kankakee, Ill.), and human albumin prepared by ethanol fractionation (E. R. Squibb & Sons, New York) or by starch block electrophoresis of fresh human serum(8) were used as reagents. Fresh human serum, and lyophilized serum (Pentex, Inc., Kankakee, Ill.) were also used. "Buffered-Agar" consisted of 0.7% Agar-Noble (Difco Lab., Detroit, Mich.) in glycine (25 g/l), sodium barbital (8.25 g/l) buffer at pH 7.7. Sodium chloride (8.5 g/l) and merthiolate (1:10,000 parts by weight) were added. For study of conditions of precipitation, the pH and salt concentration of the agar were varied, and sodium barbital and glycine were omitted.

Diffusion ring test was performed in Petri dishes filled with agar containing varied concentrations of hemoglobin. The agar medium was first melted, then cooled to 50°C; hemoglobin was then added, the medium mixed, and the plate immediately poured and cooled. Wells 0.6 cm in diameter were then cut out of the gel, and serum or albumin samples (0.15 ml) were pipetted in. After 16 to 20 hours at room temperature, the outer diameters of the resulting rings of precipitation which formed around the wells were measured with calipers from their images pro-

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