In vitro Exposure of Rabbit Ova to Estrogens.* (28929)

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It is now well established that the exposure of sub-mammalian embryos to sex hormones may result in abnormalities of the gonads, and even in sex reversal (for review see Burns(1); Pincus and Erickson(10)). The treatment with sex steroids of the young opposum, which is born at an earlier stage of development than most mammals, has also been found to result in sex reversal(1). In general, however, the effects of sex steroids on the development of the mammalian gonad remain obscure because of the *in utero* position of the embryo during embryogenesis of the gonads. Thus, it is not known to what degree the embryo has been exposed to the steroid before the steroid is inactivated or excreted by the mother.

The present report describes a series of experiments in which 1-day ova of the rabbit were removed from the reproductive tract of the mother, exposed *in vitro* to certain sex steroids, and then returned to the reproductive tract of a pseudopregnant rabbit for completion of their development. Other authors have studied the effects on viability and in some cases on development of *in vitro* exposure to various specific temperatures (Chang(2); Kardymowicz(8)), to various incubation media for storage (Gates and Runner(4); Kardymowicz(8); Hafez(6)), to antibodies (Kurosaki, Sakuma, and Mori(9); Hafez(6,7)), and to X-irradiation (Glass and Lin(5)). The present report is believed to be the first in which there has been observed a specific effect on the development process caused by *in vitro* exposure of ova to an agent.

Methods and materials. Female rabbits which had been mated with normal males one day earlier were killed with sodium pentobarbital and the Fallopian tubes were immediately removed. The tubes were flushed with a medium consisting of 5% rabbit serum in calcium-free Ringer's solution, and the ova were collected in a micropipette under a dissecting microscope. Most of the ova were at the 2-cell stage, but occasional ova were at the 4-cell stage.

In one series of experiments the ova were incubated at room temperature for 5 minutes in a microsuspension of the steroid hormone in Steroid Suspending Vehicle.† In the later experiments ova were incubated for 24 hours at 10°C in a microsuspension of the steroid hormone in a solution of 50% rabbit serum in calcium-free Ringer's solution.

Upon completion of the incubation period, the ova were transferred to the Fallopian tubes of rabbits which had been made pseudopregnant by intravenous injection of 50 international units of chorionic gonadotrophin (Follutein, E. R. Squibb and Sons, New York). Induction of pseudopregnancy was coordinated with the age of the ova when removed from the rabbit, so that 1-day ova were transferred to rabbits which were 1-day pseudopregnant. In each experiment all the ova obtained from one rabbit were transferred to the pseudopregnant recipient, divided between the 2 Fallopian tubes. The pseudopregnant recipient was anesthetized with sodium pentobarbital, and the ovary and upper part of the Fallopian tube of each side were exposed through small incisions in the flanks. Ova were introduced into the Fallopian tubes through the fimbria.

The young rabbits were recovered at birth or by Caesarian section on the 30th day of pregnancy. Laparotomy of the young rabbits exposed the reproductive tracts which were examined under a dissecting microscope for observation of anatomical sex. The gonads were fixed in Bouins and stained in hematoxylin and eosin.

Results. The results of a series of experiments in which ova were incubated for 5 min-

* Aided by a grant from the National Foundation.

† Sodium chloride 9 g, Polysolvate 80 4 ml, Carboxymethylcellulose 5 g, benzyl alcohol 9 ml, and H_2O 1000 ml.

TABLE I. Transplantation of 1-Day Rabbit Ova Following Incubation with Estrogens or Control Media at Room Temperature for 5 Minutes.

Treatment	No. ova transplanted	No. offspring	Male	Female
5% serum Ringer's	9	3	1	2
Steroid suspending vehicle	6	4	1	3
Estradiol, .1 mg/ml	33	10	7	3
Estradiol, .01 mg/ml	14	5	4	1
Stilbesterol, .1 mg/ml	41	11	6	4
Stilbesterol, .01 mg/ml	21	10	5	5
Total	124	43	24	18

utes at room temperature in steroid-free media and in media containing estradiol and stilbesterol are shown in Table I. In this series of experiments 35% of the transferred ova completed their development. Incubation in Steroid Suspending Vehicle with 0.1 mg/ml estradiol, 0.01 mg/ml estradiol, 0.1 mg/ml stilbesterol or 0.01 mg/ml stilbesterol had no apparent effect on the recovery of live offspring. There appeared to be no significant deviation from the expected sex ratio, and dissection of these young rabbits to reveal their reproductive tracts showed no apparent abnormalities. In a similar series of experiments carried out on 6-day blastocysts no effect of the treatment could be observed.

The lack of effect of exposure to high concentrations of estradiol and stilbesterol suggested that exposure for 5 minutes at room temperature did not result in significant penetration of the steroids into the cells of the ova. Since the diffusion of steroid into cells would be expected to have a Q_{10} of approximately 1, it was decided to extend the exposure time to 24 hours and to lower the exposure temperature to 10°C. Chang(3) has shown that ova stored for 24 hours at 10°C were able to develop when returned to the reproductive tract, and that the sex ratio of rabbits developing from these ova was not altered. As indicated in Table II, when ova were exposed to serum Ringer's solution without steroid added for 24 hours at 10°C, 33% developed into young rabbits on return to the reproductive tract. When estradiol at a concentration of either 1 mg/ml or 0.1 mg/ml, or stilbesterol at a concentration of 1 mg/ml was added, no ova developed. A small percentage of ova developed after being exposed to stilbesterol at a concentration of 0.1 mg/ml. Of 404 ova exposed to 0.1 mg/ml of stilbesterol for 24 hours at 10°C, 24 survived to birth. These included 4 normal appearing males and 17 normal appearing females. The sex of the remaining 3 animals was unclear upon gross examination at laparotomy, since the position of the gonads was intermediate between that of the male and that of the female. The gonadal tissue of these 3 individuals was determined as testicular on histological examination. The gonadal tissue of one of these 3 individuals is compared in Fig. 1 with that of a normal male of the same age. It can be seen that the differentiation into tubules is retarded in the gonad of the steroid treated male.

Discussion. The present experiments indicate that the exposure of 1-day rabbit ova to high concentrations of estrogens is lethal to them. This lethality may be non-specific and have little relationship to the estrogenic ac-

TABLE II. Transplantation of 1-Day Rabbit Ova Following Exposure to Estrogens and Control Media for 24 Hours at 10°C.

Treatment	No. ova transplanted	No. offspring	% offspring	Male	Female	Unknown*
Control—No steroid	27	9	33	†	†	
Estradiol, 1 mg/ml	16	0	0		—	
Estradiol, .1 mg/ml	35	0	0	—	—	
Stilbesterol, 1 mg/ml	47	0	0	—	—	
Stilbesterol, .1 mg/ml	404	24	5.9	4	17	3

* Not possible to determine sex by observation of reproductive organs at laparotomy.
† Not observed—See text.

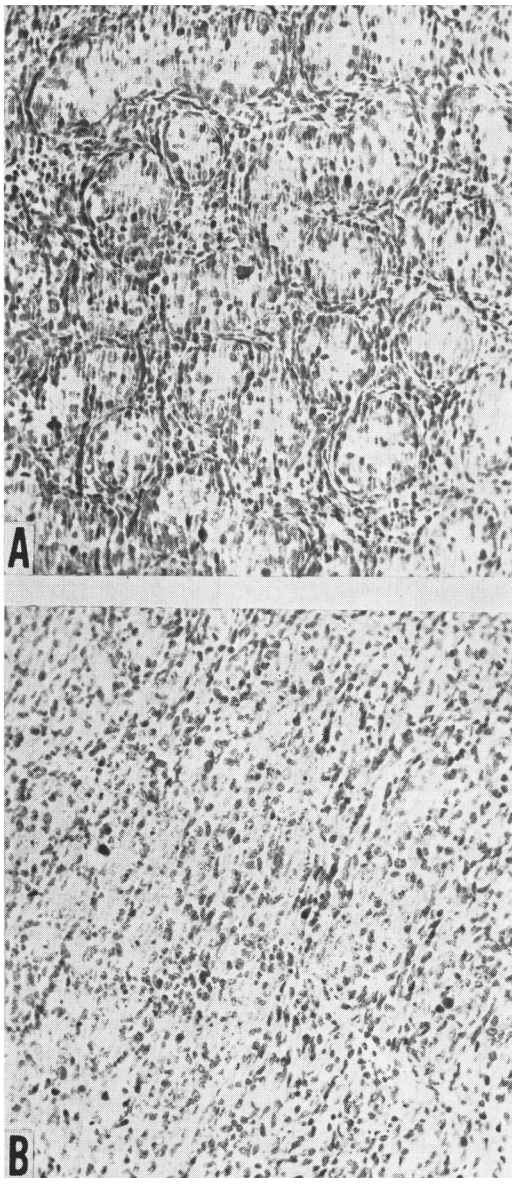

FIG. 1. Gonads from male rabbits on 30th day of gestation. Hematoxylin and eosin. A. Normal male. B. Male which as a 1-day ovum was exposed to 0.1 mg/ml of stilbesterol for 24 hr at 10°C.

tivity of the steroids, although further experiments will be required to clarify this point.

There is also an indication that certain of the ova which survived treatment with stilbesterol developed into abnormal males. Since visible differentiation occurred sometime after these ova were exposed to the steroid, it must be supposed either that the steroid remained in the developing ovum until the time of differentiation of the gonads, or that the steroid affected the 1-day ova in a manner that influenced the later differentiation of the gonads.

Summary. One-day ova of the rabbit were removed from the reproductive tract of the mother, exposed *in vitro* to certain sex steroids, and then returned to the reproductive tract of a pseudopregnant rabbit for completion of their development. Ova incubated for 5 minutes at room temperature in 0.1 mg/ml estradiol, 0.01 mg/ml estradiol, 0.1 mg/ml stilbesterol or 0.01 mg/ml stilbesterol survived as well as control ova incubated without steroid, and no abnormalities of the reproductive tract were observed. When the incubation period with the steroid was extended to 24 hours at a temperature of 10°C, 1 mg/ml estradiol, 0.1 mg/ml estradiol, and 1 mg/ml stilbesterol were lethal to the ova. Of 404 ova treated with 0.1 mg/ml stilbesterol for 24 hours at 10°C, 24 young rabbits were recovered. These included 17 normal females, 4 normal males, and 3 animals whose gonads consisted of testicular tissue without normal tubule formation, and whose gonads were in an intermediate anatomical position between that of normal males and females.

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Received September 23, 1963. P.S.E.B.M., 1964, v115.