In theory, it is best to select and isolate a single cell and to derive a cell from it. This is relatively simple for most heteroploid cells but very difficult for diploid cells. Larger clones have a better chance to grow into cell lines. This population effect demonstrates the need for suspending the newly isolated clone in as small a volume of medium as is practical.

Methods of increasing efficiency. This paper reports higher plating efficiencies for diploid cells than generally reported. The reason for this is not clear. The answer may lie in controlling conditions, including media, temperature, CO_2, pH, and humidity. There is also a possibility that there is a time element involved. If there is a 5-minute delay between the preparation of a cell dilution and its inoculation, the cell concentration in the dilution tube will be greatly diminished because diploid cells attach to glass very rapidly. In some cases, we have observed 50% of the cells attach within 5 minutes. To offset this, one person counts the cells while simultaneously a second makes the dilution and plants the cells. In this way the calculated number of cells is more closely related to the number actually planted.

The variation of plating efficiency of from 5%-100% for heteroploid and from 5%-50% for diploid cells cannot be due to variations in environment because each experiment had a HeLa S3 control. The variations may be due to differences in the condition of the cells when they were planted or they may represent differences between cell types.

Summary and conclusions. A method which appears a practical and easy way to produce cloned cell lines is presented, with some observations and problems to be considered when cloning. Although the method attempts to derive cell lines from single cells by taking many precautions, it cannot be definitely stated that this goal is actually accomplished. It has yet to be demonstrated satisfactorily that a single diploid cell can in fact be cloned. The method, however, definitely produces cell lines which are derived from such a reduced population that morphological differences cannot be demonstrated within a clone, but can be demonstrated between cells from different clones.

1. Sanford, K. K., Covalesky, H. B., Dupree, L. T., Earle, W. R., *Exp. Cell. Research,* 1961, v23, 361.
2. Lwoff, A., Dulbecco, R., Vogt, Marguerite, Lwoff, Marguerite, *J. Exp. Med.,* 1956, v104, 615.
3. Puck, T. T., Marcus, P. I., Cieciura, S. J., *ibid.,* 1956, v103, 273.
4. Schenck, M., Moskowitz, M., PROC. SOC. EXP. BIOL. AND MED., 1955, v99, 30.
5. Puck, T. T., Cieciura, S. J., Fisher, H. W., *J. Exp. Med.,* 1957, v106, 145.
6. Pious, Donald A., Hamburger, Robert N., Mills, Stanley E., *Fed. Proc.,* 1963, v22, 382.

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Ethynyl Estradiol: A Potent Orally Active Contraceptive in Rats. (29243)

A. S. WATNICK, J. GIBSON, M. VINEGRA,* AND S. TOLKSDORF
(Introduced by P. L. Perlman)
Endocrine Research, Department of Biological Research, Schering Corporation, Bloomfield, N. J.

The action of estrogens in producing a state of infertility in a large number of animal species has been well documented. Smith (1) demonstrated that estrin, an ovarian extract, produced sterility in rats. Pincus and Kirsch(2) obtained similar results when treating rabbits with estrone. Brown and coworkers(3) were able to inhibit ovulation in the human with daily doses of stilbestrol. More recently, ethynyl-estradiol- 3 methyl-ether, without addition of a progestagen, has been reported by Walker to be an effective oral contraceptive in humans (cited by Jackson; 4). The contraceptive action of estro-

* Present address: Seton Hall School of Medicine and Dentistry, Jersey City, N. J.

gens can be due to any of the following biological activities: A) inhibition of pituitary gonadotropin release(5,6), B) interference with ova transport(7), and C) prevention of nidation(8).

The present study was conducted to determine the mode of contraceptive action of ethynyl estradiol (EE) at the minimum dose required to prevent pregnancy in 100% of the treated rats (MPD_{100}). To correlate results of the various studies more easily, all experiments were conducted with animals of the same strain, sex, and weight.

Materials and methods. Adult Long-Evans rats (Rockland Farms, New City, N. Y.) weighing 150-175 g were used. They were housed in a room providing alternating 12 hours of light and 12 hours of darkness and were permitted food and water *ad libitum*.

A. *12-day mating test.* Ethynyl estradiol (17α-ethynyl-1,3,5(10)-estratrien-3,17β-diol) contained in 1 cc of a carboxymethylcellulose solution was given by gavage for 11 days to mature female rats. During that period they were housed with males at a ratio of 3 females to one male. Control animals received the suspending medium only (p.o.). On the twelfth day the female animals were autopsied and the number of pregnancies determined by counting the number of nidation sites.

B. *45-day mating test.* The procedure used was essentially that used in A above. However, in addition to the extended treatment period, daily vaginal smears were performed to determine whether the animal mated as well as to see the effect of EE on the estrous cycle. At the end of the treatment period the animals were autopsied and the ovaries, uteri, pituitaries, thyroid, thymus, liver, and kidneys excised and their weights recorded. The ovaries were placed in 10% neutral formalin for histological sectioning and staining.

C. *Compensatory ovarian hypertrophy in the unilaterally ovariectomized rat.* Female rats were randomly selected and the right ovary removed while under light ether anesthesia. Immediately upon recovery oral administration with EE was started and continued daily for 11 days. Twenty-four hours after the last treatment the rats were autopsied and the remaining ovary and the uteri from each animal were excised and weighed. Ovaries and uteri from intact, vehicle-treated females served as controls.

TABLE I. Effect of Various Doses of Ethynyl Estradiol in Mating Test.

Compound	Dose (mg/kg)	No. of animals	% pregnant	Avg No. of sites
Control	—	12	83	11
Ethynyl estradiol	.5	16	0	0
Idem	.05	16	0	0
"	.005	16	50	5

D. *"Time studies."* Female rats were placed with males of known fertility (ratio, 3:1, respectively). Daily vaginal smears were inspected for presence of sperm. Those females which had mated the previous evening were considered to be in the first day of pregnancy. Oral treatment with EE was started on either the first, second, third, fourth, or fifth day of pregnancy and continued until the eleventh day. Twenty-four hours after the last gavage the rats were autopsied and the number of nidation sites counted.

E. *Inhibition of the decidual response.* Rats in estrus or late proestrus were made pseudopregnant by stimulation of the cervix with a faradic current. Four days later one horn of the uterus was scratched along the antimesometrial wall. Oral administration with EE was begun immediately and continued for 3 days. Twenty-four hours after the last treatment the animals were sacrificed and the traumatized horn of the uterus excised and weighed. The weight of the contralateral horn served as a control.

Results. The minimum protective dose (MPD_{100}) was defined as the lowest concentration of a compound which prevented pregnancy in *all* the animals when given orally. The MPD_{100} of EE was found to be approximately 0.05 mg/kg body weight per day (Table I).

The MPD_{100} of EE proved to be as effective a contraceptive during the 45 days of treatment as it was for 11 days. This was indicated by the fact that none of the 9 animals used in this study delivered during the test period nor was any animal found to contain nidation sites at time of autopsy.

TABLE II. Organ Weights of Females Treated 45 Days with the MPD_{100} of Ethynyl Estradiol.

Compound	No. of animals	Body wt ± S.D.	Organ wt/mg/100 g body wt ± S.D.							
			Ovaries	Uterus	Adrenals	Pituitary	Thyroid	Thymus	Liver	Kidneys
									(g/100)	
Control	5	224 ±16.7	48.7 ±3.7	137.6 ±21.9	27.2 ±16.1	4.4 ±1.4	6.9 ± .03	149.4 ±26.8	4.2 ±.66	774 ±51
Ethynyl estradiol (.05 mg/kg)	9	224 ±38.7	36.3† ±9.7	171.7 ±69.8	28.1 ± 3.8	4.7 ± .99	6.8 ±1.60	98.2* ±21.7	4.4 ±.59	750 ±93

* P = .01

† P = .02

There was no set pattern in length of the estrous cycle in the EE-treated rats. Some cycles were longer and others were shorter than what is usually considered normal in this animal strain (4.5-5 days). In spite of the fact that an estrogen was being administered, only one female exhibited a period of constant estrus and this lasted for only 5 days.

The data also indicate that the sexual receptivity of 4 of the animals was increased, especially one rat which mated 11 times in 15 days. The other 3 animals accepted the male for at least 4 consecutive days. It was apparent that with this treatment the vaginal smear could not be used to predict whether the female would accept the male, for in many instances the rats mated when their vaginal smear indicated they were in a diestrous state.

Organ weights of the females at autopsy showed a decrease in the average ovarian and thymus weights as well as a slight increase in average uterine weights (Table II). The histology of the ovaries from these animals showed a decrease in number of maturing follicles. The ovaries had many corpora lutea which showed no sign of degeneration.

The results (Table III) indicate that 12 days after unilateral ovariectomy there is approximately a 45% increase in weight of the remaining ovary. At 0.1, 0.05, and 0.025 mg/kg body weight (per day, p.o.), EE prevented this compensatory ovarian hypertrophy.

In the "Time Study," 14 of the 15 untreated control rats contained nidation sites on the 12th day (Table IV). On the other hand, 0.05 mg/kg body weight of EE, which was totally effective when given before mating, had a minimal effect in preventing pregnancy when given *after* coitus.

The most pronounced action of the MPD_{100} of EE was in causing fetal death. The largest number of deaths was caused when treatment was started on the first day of pregnancy (92% of the pregnant animals showed signs of aborting). When treatment was delayed until a later day, the effect was not as great although significant numbers of females were aborting.

The results (Table V) show that at the 3 dose levels used, 0.075, 0.050, and 0.025 mg/kg, there was no inhibition of the decidual response.

Discussion. From these observations it is evident that ethynyl estradiol at comparatively low concentrations will prevent pregnancy in the rat when treatment is initiated before coitus. Inhibition of pituitary gonadotropin release may well be the mode of contraceptive action. At least in this study it was the only measurable activity observed at

TABLE III. Effect of Ethynyl Estradiol (EE) in Unilaterally Ovariectomized Rats.

Group	Treatment (mg/kg)	No. of rats	Body wt (g)	Ovary wt (mg) Left*	Right	Uterine wt (mg)
Intact	—	7	211 ± 15.5†	48.5 ± 7.6†	51.9 ± 7.2†	239 ± 46.8†
Unilaterally ovariectomized	—	7	211 ± 11.7	70.1 ± 15.5	—	288 ± 63.5
"	EE .1	7	192 ± 7.2	43.9 ± 4.9	—	265 ± 44.1
"	EE .05	7	182 ± 15.5	41.7 ± 3.7	—	255 ± 43.7
"	EE .025	7	187 ± 22.3	47.2 ± 12.2	—	188 ± 42.1

* A significant difference was found between avg ovarian weights of intact and untreated unilaterally ovariectomized group as well as EE treated unilaterally ovariectomized group and untreated unilaterally ovariectomized group ($p = .01$).
† ± S.D.

TABLE IV. Effect of MPD_{100} Dose (0.05 mg/kg) of Ethynyl Estradiol in "Time Studies."

Day of pregnancy treatment started	No. of animals	% pregnant on day 12	Avg No. of sites/pregnant animal	% of pregnant animals showing signs of resorption
—	15	94	10.6	0
1	14	86	7.1	92
2	11	82	9.0	33
3	11	64	8.9	57
4	13	92	9.6	67
5	12	92	10.6	33

TABLE V. Effect of Ethynyl Estradiol on the Decidual Response in the Intact Rat.

Compound	Dose (mg/kg)	No. of animals	Final body wt (g)	Avg wt (mg) Control uterus	Traum. uterus
Control	—	6	193 ±22.8*	138 ± 30.9*	820 ± 485*
Ethynyl estradiol	.075	"	182 ± 8.3	127 ± 28.2	965 ± 551
" "	.05	"	184 ± 5.6	132 ± 23.2	774 ± 293
" "	.025	"	197 ± 5.0	144 ± 20.3	790 ± 255

* ± S.D.

the MPD_{100}. Because of the relative insensitivity of the pituitary gonadotropin assay, it is not possible to determine which of the gonadotropins was primarily affected. EE at the MPD_{100} could conceivably inhibit the release of both Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). Dorfman and Dorfman(9) using the parabiotic rat demonstrated that estrogens can prevent the release of FSH. McCann and Taleisnik(10) have shown that estrogens and estrogens plus progestins can inhibit the release of LH.

Estrogens have long been known to be capable of maintaining the life of the corpus luteum(11,12). The existence of corpora lutea has been demonstrated histologically in our ethynyl estradiol-treated rats. Thus, the progesterone produced by the corpora lutea may act with the ethynyl estradiol to inhibit LH. Studies are now being conducted to determine if these corpora lutea are functional. Along similar lines, Nicoll and Meites(13) found that pituitaries from estrogen-treated rats produce large amounts of prolactin, which is luteotropic in the rat.

Indirect evidence which points to pituitary gonadotropin inhibition (blockade of ovulation) as the contraceptive action of EE at the MPD_{100} is obtained from the results of the "Time Studies." The animals in this experiment were treated after ovulation had taken place but during the time of ova migration through the oviducts and subsequent implantation in the uterus. The presence of nidation sites, found when the animals were

in their 12th day of pregnancy, indicated that EE could not have interfered with ova transport or ova implantation. The fact that the decidual reaction was not influenced at doses related to the MPD_{100} lends additional proof that nidation is not affected.

There are many effects associated with estrogen treatment which could have caused resorption of the fetuses in the "Time Studies." The destruction of the developing placenta and/or impairment of the migrating ova could be responsible. Schofield(14) has obtained evidence that in the rabbit estrogens may inhibit the production of progesterone by the placenta.

We have seen that the MPD_{100} of EE increased the sexual receptivity of some of the females. Austin and Bruce(15) using sufficient stilbestrol to produce a state of infertility and constant estrus, found that rats still mated but at intervals which approximated their normal periods of heat (every 4.5-5 days). On the other hand, our rats displayed no cyclic characteristic in their mating response, the frequency being increased in some and decreased in others. The decrease in number of times the rat came into heat could be due to the act of coitus and/or maintenance of the corpora lutea by the estrogen treatment, either action causing a state of pseudopregnancy. The increased receptivity displayed by the other rats might result from EE stimulation of a hypothalamic center controlling libido. This center has been postulated to exist by Brookhart *et al* (16) and Sawyer and Robison(17). More recently, Barraclough and Gorski(18), using androgen-sterilized constant estrus rats, have obtained additional evidence to support this theory. The marked difference in the frequency of mating in our rats could be due to their individual "Mating Center" sensitivities to the estrogen treatment.

Summary. The minimum protective dose of ethynyl estradiol (EE) which prevented pregnancy in all female rats treated before mating (MPD_{100}) was found to be 0.05 mg/kg body weight. This concentration of EE appeared to increase the sexual receptivity of some of the females. In attempting to clarify the mode of contraceptive action at the MPD_{100}, EE was found to inhibit pituitary gonadotropin release but not ova transport or ova nidation. This was concluded from the facts that EE inhibited compensatory ovarian hypertrophy of the unilateral ovariectomized rat but did not prevent implantation when given after coitus, although a high rate of resorption was noted. In addition, the MPD_{100} of EE did not inhibit formation of deciduomata.

1. Smith, M. G., *Bull. Johns Hopkins Hosp.,* 1926, v39, 203.

2. Pincus, G., Kirsch, R. E., *Am. J. Physiol.,* 1936, v115, 219.

3. Brown, W. E., Bradbury, J. T., Jennings, A. F., *J. Clin. Endocrinol.,* 1948, v8, 453.

4. Jackson, M. C. N., *J. Reprod. Fertil.,* 1963, v6, 153.

5. Halpern, S. R., D'Amour, F. E., *Am. J. Physiol.,* 1936, v115, 229.

6. Perlman, P. L., Thomas, G. B., Cassidy, J. W., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 158.

7. Whitney, R., Burdick, M. O., *Endocrinol.,* 1936, v20, 643.

8. Velardo, J. T., Hisaw, F. L., *ibid.,* 1951, v49, 530.

9. Dorfman, R. I., Dorfman, A. S., *ibid.,* 1963, v72, 115.

10. McCann, S. M., Taleisnik, S., *ibid.,* 1961, v69, 909.

11. Westman, A., Jacobsohn, D., *Acta Obst. Gynec. Scandinav.,* 1937, v17, 1.

12. Merckel, C., Nelson, W. O., *Anat. Rec.,* 1940, v76, 391.

13. Nicoll, C. S., Meites, J., *Endocrinol.,* 1962, v70, 272.

14. Schofield, B. M., *ibid.,* 1962, v25, 95.

15. Austin, C. R., Bruce, H. M., *ibid.,* 1956, v13, 376.

16. Brookhart, J. M., Dey, F. L., Rawson, S. W., *Endocrinol.,* 1941, v28, 561.

17. Sawyer, C. H., Robison, B., *J. Clin. Endocrinol.,* 1956, v16, 914.

18. Barraclough, C. A., Gorski, R. A., *J. Endocrinol.,* 1962, v25, 175.

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